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<b>(54) Title:</b> TARGETED GENE DELIVERY TO CELLS BY FILAMENTOUS BACTERIOPHAGE  <b>(57) Abstract</b>  This invention provides methods specifically delivering and expressing heterologous nucleic acids in target cells. The methods generally involve providing a phage externally displaying a heterologous targeting protein (e.g., a scFv) and containing a heterologous nucleic acid; and contacting the target cell with the said phage whereby the phage is internalized into the cell and wherein the heterologous nucleic acid is transcribed, and optionally translated, within the cell.		

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## TARGETED GENE DELIVERY TO CELLS BY FILAMENTOUS BACTERIOPHAGE

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit under 35 U.S.C. §119(e) of provisional application USSN 60/082,953, filed on April 24, 1998, which is herein incorporated by reference in its entirety for all purposes.

### STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This work was supported, in part, by Department of Defense Grants DAMD17-96-1-6244 and DAMD17-94-4433. The government of the United States of America may have some rights in this invention.

### FIELD OF THE INVENTION

This invention relates to the field of cell transduction and gene delivery. In particular, this invention relates to the use of filamentous phage to deliver heterologous nucleic acids into a cell.

### BACKGROUND OF THE INVENTION

Frequently gene transfection techniques require the ability to target a therapeutic gene to an appropriate "target" cell or tissue type with high efficiency (Michael and Curiel (1994) *Gene Ther.* 1: 223-232). Targeting of retroviral vectors has been reported by inserting receptor ligands or single chain Fv (scFv) antibody fragments into the viral envelope protein (Kasahara *et al.* (1994) *Science* 266: 1373-1376). Targeting of adenoviral vectors has been achieved by use of 'adapter' fusion molecules consisting of an antibody fragment which binds the adenoviral knob and a cell targeting molecule such as a receptor ligand or antibody (Douglas *et al.* (1996) *Nat. Biotechnol.* 14: 1574-1578; Watkins *et al.* 1997) *Gene Ther.* 4(10): 1004-1012). Targeting of non-viral vectors using cell surface receptor ligands or antibodies has also been reported (Fominaya and Wels (1996) *J. Biol. Chem.* 271(18): 10560-10568; Michael and Curiel (1994) *Gene Ther.* 1: 223-232). All of

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these approaches depend on the use of targeting molecules that bind a cell surface receptor resulting in internalization of the gene delivery vehicle with subsequent delivery of the DNA to the nucleus.

Identification of appropriate targeting molecules has largely been performed by individually screening receptor ligands or antibodies. In the case of single chain (*e.g.* scFv) antibody fragments this typically requires construction of the scFv from the V-genes of a hybridoma, construction of the targeted gene delivery vehicle, and *in vitro* evaluation of targeting ability.

More recently, it has proven possible to directly select peptides and antibody fragments binding cell surface receptors from filamentous phage libraries (Andersen *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93(5): 1820-1824; Barry *et al.* (1996) *Nat. Med.* 2: 299-305; Cai and Garen (1995) *Proc. Natl. Acad. Sci. USA* 92(24): 6537-6541; de Kruif *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92(6): 3938-3942; Marks *et al.* (1993) *Bio/Technology* 11(10): 1145-1149). This has led to a marked increase in the number of potential targeting molecules.

Despite the increase in the number of known potential targeting molecules, these molecules, to date, have not been effectively exploited for transfecting genes into target cells.

## SUMMARY OF THE INVENTION

This invention is based, in part, on the discovery that filamentous phage displaying the an antibody that binds to an internalizing receptor (*e.g.* anti-ErbB2 scFv F5) as a genetic fusion with the phage minor coat protein pIII can directly infect mammalian cells expressing the target receptor epitope (*e.g.*, ErbB2) leading to expression of a heterologous gene (*e.g.* cDNA) contained in the phage genome. Thus, in one embodiment, this invention provides methods of transfecting (transducing) a target cell (*e.g.*, vertebrate, invertebrate, bacteria, fungus, yeast, algal cell) with a (*e.g.* heterologous) nucleic acid. The methods preferably involve i) providing a phage externally displaying a heterologous targeting protein (heterologous to the phage) and containing a heterologous nucleic acid (heterologous to the phage and/or to the target cell); and ii) contacting the target cell with the phage whereby said phage is internalized into said cell and wherein the heterologous nucleic acid is transcribed within the cell. While in many embodiments, the heterologous

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nucleic acid comprises a reporter gene (or cDNA) or a selectable marker (*e.g.* an antibiotic - resistance gene or cDNA), in particularly preferred embodiment, the heterologous nucleic acid transcribes a gene product (*e.g.*, antisense molecule, ribozyme, polypeptide) other than, or in addition to, the reporter gene or selectable marker. Typically a DNA brought into the cell by the methods of this invention is single stranded and, without being bound to a particular theory, it is believed the DNA is replicated to double stranded prior to transcription.

In one preferred embodiment, the phage used in the methods of this invention are monovalent, displaying, on average, one pIII fusion protein per viral particle, while in other preferred embodiments, the phage used in the methods of this invention are multivalent, displaying on average, at least two, more preferably at least 3, and most preferably at least 5, pIII fusions per viral particle. The phage used to deliver the heterologous nucleic acid into a target cell can be a member of a library of phage wherein said library comprises a number of different heterologous targeting proteins (*e.g.* containing, on average, at least  $10^5$ , preferably at least  $10^6$ , more preferably at least  $10^7$ , and most preferably at least  $10^8$  different members). The methods can further involve selecting phage (*e.g.*, from a library) that are internalized by the target cell. The selection can be by a variety of means including, but not limited to detection of a reporter gene (*e.g.* GFP, Fflux, luciferase,  $\beta$ -gal, *etc.*) or by selection via a selectable marker (*e.g.* an antibiotic resistance gene). The method can further involve amplifying phage internalized by said cell.

In one particularly preferred embodiment, the providing step involves i) providing an assembly cell containing the heterologous nucleic acid and a packaging signal; and ii) infecting the assembly cell with a phage expressing on its surface said heterologous targeting protein and containing the gene for the targeting protein whereby the phage acts as a helper phage and packages the heterologous nucleic acid. Preferred assembly cells are prokaryotic cells (*e.g.* bacterial cells). In one preferred embodiment the heterologous targeting protein, and/or a DNA encoding the heterologous targeting protein, and/or the heterologous nucleic acid are encoded by a DNA that is a phagemid. Preferred phage for use in the methods of this invention are filamentous phage.

Preferred heterologous targeting proteins are antibodies, more preferably single-chain Fv, or Fabs. The phage can be preselected for binding to a particular internalizing cell surface receptor (*e.g.*, erbB2). Other preferred receptors include, but are

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not limited to receptors for platelet-derived growth factor (PDGF), epidermal growth factor-  
(EGF), insulin-like growth factor (IGF), transforming growth factor  $\beta$  (TGF- $\beta$ ), fibroblast  
growth factors (FGF), interleukin 2 (IL2), nerve growth factor (NGF), interleukin 3 (IL3),  
interleukin 4 (IL4), interleukin 1 (IL1), interleukin 6 (IL6), interleukin 7 (IL7), interleukin  
5 13, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-  
stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), erythropoietin  
TGF, transferrin, erbB2, EGF, Vegf, and the like. In one particularly preferred embodiment,  
the phage can further express an endosomal escape polypeptide and/or a nuclear localization  
signal.

10 In another embodiment, this invention provides a vector for (*e.g.*, specific)  
transfection of a target cell. Preferred vectors comprise a phage displaying a heterologous  
targeting protein (*e.g.* a single chain antibody) that specifically binds to an internalizing  
receptor whereby the phage binds to and is internalized into the target cell, and wherein the  
phage contains a heterologous nucleic acid that is transcribed inside the target cell. In one  
15 preferred embodiment, the heterologous nucleic acid transcribes a gene product (*e.g.*,  
antisense molecule, ribozyme, polypeptide) other than, or in addition to, a reporter gene or  
selectable marker. The vector can include any of the viral particles or nucleic acids encoding  
the viral particles, or cells containing the nucleic acid or viral particles described herein.

Thus, in another preferred embodiment this invention comprises a phage  
20 vector or phagemid vector encoding: a phage coat protein in fusion with a heterologous  
targeting protein that specifically binds to an internalizing cell surface receptor and is  
internalized into a cell bearing said receptor; and a heterologous nucleic acid in an  
expression cassette allowing transcription of the heterologous nucleic acid inside said cell as  
described herein. In one preferred embodiment, the heterologous nucleic acid transcribes a  
25 gene product (*e.g.*, antisense molecule, ribozyme, polypeptide) other than, or in addition to, a  
reporter gene or selectable marker

This invention also provides a kit for transducing a target cell. The kit  
preferably a phage, and/or phage DNA, and/or phagemid DNA, and/or cell(s) containing  
phage and/or phagemid DNA, and/or cells containing phage particles as described herein. In  
30 on particular preferred embodiment, the kits include a phage or phagemid vector encoding:  
a phage coat protein in fusion with a heterologous targeting protein that specifically binds to  
an internalizing cell surface receptor and is internalized into a cell bearing said receptor; and

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a pair of restriction sites that allow insertion of a heterologous nucleic acid into the phage or phagemid vector. The restriction sites are preferably situated in an expression cassette such that a gene or cDNA inserted between said restriction sites is operably linked to a promoter and is transcribed, and optionally translated, when said expression cassette is transduced into a target cell.

### DEFINITIONS

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $F(ab)_2$ , a dimer of Fab which itself is a light chain joined to  $V_H-C_H1$  by a disulfide bond. The  $F(ab)_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the  $(Fab)_2$  dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see, *Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Preferred

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antibodies include single chain antibodies (antibodies that exist as a single polypeptide chain), more preferably single chain Fv antibodies (scFv or scFv) in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked  $V_H$ - $V_L$  heterodimer which may be expressed from a nucleic acid including  $V_H$ - and  $V_L$ - encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, *et al.* (1988) *Proc. Nat. Acad. Sci. USA*, 85: 5879-5883. While the  $V_H$  and  $V_L$  are connected to each as a single polypeptide chain, the  $V_H$  and  $V_L$  domains associate non-covalently. The first functional antibody molecules to be expressed on the surface of filamentous phage were single-chain Fv's (scFv), however, alternative expression strategies have also been successful. For example Fab molecules can be displayed on phage if one of the chains (heavy or light) is fused to g3 capsid protein and the complementary chain exported to the periplasm as a soluble molecule. The two chains can be encoded on the same or on different replicons; the important point is that the two antibody chains in each Fab molecule assemble post-translationally and the dimer is incorporated into the phage particle via linkage of one of the chains to g3p (*see, e.g.*, U.S. Patent No: 5733743). The scFv antibodies and a number of other structures converting the naturally aggregated, but chemically separated light and heavy polypeptide chains from an antibody V region into a molecule that folds into a three dimensional structure substantially similar to the structure of an antigen-binding site are known to those of skill in the art (*see e.g.*, U.S. Patent Nos. 5,091,513, 5,132,405, and 4,956,778). Particularly preferred antibodies include all those that have been displayed on phage I think preferred antibodies should include all that have been displayed on phage (*e.g.*, scFv, Fv, Fab and disulfide linked Fv (Reiter *et al.* (1995) *Protein Eng.* 8: 1323-1331).

An "antigen-binding site" or "binding portion" refers to the part of an immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions" or "FRs". Thus, the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed



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relative to each other in three dimensional space to form an antigen binding "surface". This surface mediates recognition and binding of the target antigen. The three hypervariable regions of each of the heavy and light chains are referred to as "complementarity determining regions" or "CDRs" and are characterized, for example by Kabat *et al.* Sequences of proteins of immunological interest, 4th ed. U.S. Dept. Health and Human Services, Public Health Services, Bethesda, MD (1987).

As used herein, the terms "immunological binding" and "immunological binding properties" refer to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength or affinity of immunological binding interactions can be expressed in terms of the dissociation constant ( $K_d$ ) of the interaction, wherein a smaller  $K_d$  represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" ( $k_{on}$ ) and the "off rate constant" ( $k_{off}$ ) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of  $k_{off}/k_{on}$  enables cancellation of all parameters not related to affinity and is thus equal to the dissociation constant  $K_d$  (see, generally, Davies *et al.* (1990) *Ann. Rev. Biochem.*, 59: 439-473).

The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, F5 or C1 antibodies can be raised to the c-erbB-2 protein that bind c-erbB-2 and not to other proteins present in a tissue sample. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically

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immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The terms "polypeptide", "peptide", or "protein" are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The amino acid residues are preferably in the natural "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. In addition, the amino acids, in addition to the 20 "standard" amino acids, include modified and unusual amino acids, which include, but are not limited to those listed in 37 CFR §1.822(b)(4). Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates either a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to a carboxyl or hydroxyl end group.

The term "binding polypeptide" refers to a polypeptide that specifically binds to a target molecule (e.g. a cell receptor) in a manner analogous to the binding of an antibody to an antigen. Binding polypeptides are distinguished from antibodies in that binding polypeptides are not ultimately derived from immunoglobulin genes or fragments of immunoglobulin genes.

The term "conservative substitution" is used in reference to proteins or peptides to reflect amino acid substitutions that do not substantially alter the activity (specificity or binding affinity) of the molecule. Typically conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (e.g. charge or hydrophobicity). The following six groups each contain amino acids that are typical conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

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The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. The term also includes peptide nucleic acids. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated.

Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19: 5081; Ohtsuka *et al.* (1985) *J. Biol. Chem.* 260: 2605-2608; and Cassol *et al.* (1992); Rossolini *et al.*, (1994) *Mol. Cell. Probes* 8: 91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The terms "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. However, the term "isolated" is not intended refer to the components present in an electrophoretic gel or other separation medium. An isolated component is free from such separation media and in a form ready for use in another application or already in use in the new application/milieu.

The term "expression cassette", refers to nucleotide sequences which are capable of affecting expression of a structural gene in hosts compatible with such sequences. Such cassettes include at least promoters and optionally, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be used as described herein.

The term "operably linked" as used herein refers to linkage of a promoter upstream from a DNA sequence such that the promoter mediates transcription of the DNA sequence.

A fusion protein is a chimeric molecule in which the constituent molecules are all polypeptides and are attached (fused) to each other through terminal peptide bonds so that the chimeric molecule is a continuous single-chain polypeptide. The various

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constituents can be directly attached to each other or can be coupled through one or more peptide linkers.

A "target" cell refers to a cell or cell-type that is to be specifically bound by a member of a phage display library or a chimeric molecule of this invention. Preferred target cells are cells for which an internalizing antibody or binding polypeptide is sought. The target cell is typically characterized by the expression or overexpression of a target molecule that is characteristic of the cell type. Thus, for example, a target cell can be a cell, such as a tumor cell, that overexpresses a marker such as c-erbB-2.

A "targeting moiety" refers to a moiety (*e.g.* a molecule) that specifically binds to the target molecule. Where the target molecule is a molecule on the surface of a cell and the targeting moiety is a component of a chimeric molecule, the targeting moiety specifically binds the chimeric molecule to the cell bearing the target. Where the targeting moiety is a polypeptide it can be referred to as a "targeting polypeptide".

The terms "internalizing" or "internalized" when used in reference to a cell refer to the transport of a moiety (*e.g.* phage) from outside to inside a cell. The internalized moiety can be located in an intracellular compartment, *e.g.* a vacuole, a lysosome, the endoplasmic reticulum, the golgi apparatus, or in the cytosol of the cell itself.

An internalizing receptor or marker is a molecule present on the external cell surface that when specifically bound by an antibody or binding protein results in the internalization of that antibody or binding protein into the cell. Internalizing receptors or markers include receptors (*e.g.*, hormone, cytokine or growth factor receptors) ligands and other cell surface markers binding to which results in internalization. ]

The term "heterologous nucleic acid" refers to a nucleic acid that is not native to the cell in which it is found or whose ultimate origin is not the cell or cell line in which the "heterologous nucleic acid" is currently found.

The idiotype represents the highly variable antigen-binding site of an antibody and is itself immunogenic. During the generation of an antibody-mediated immune response, an individual will develop antibodies to the antigen as well as anti-idiotypic antibodies, whose immunogenic binding site (idiotype) mimics the antigen. Anti-idiotypic antibodies can also be generated by immunization with an antibody, or fragment thereof,

A "phage display library" refers to a collection of phage (*e.g.*, filamentous phage) wherein the phage express an external (typically heterologous) protein. The external

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protein is free to interact with (bind to) other moieties with which the phage are contacted. Each phage displaying an external protein is a "member" of the phage display library.

An "antibody library" refers to phage display library that displays antibodies (binding proteins encoded by one or more antibody genes or cDNAs). The antibody library  
5 includes the population of phage or a collection of vectors encoding such a population of phage, or cell(s) harboring such a collection of phage or vectors. The library can be monovalent, displaying on average one single-chain antibody per phage particle or multi-valent displaying, on average, two or more single chain antibodies per viral particle. Preferred antibody libraries comprise on average more than  $10^6$ , preferably more than  $10^7$ ,  
10 more preferably more than  $10^8$ , and most preferably more than  $10^9$  different members (*i.e.* encoding that many different antibodies).

The term "filamentous phage" refers to a viral particle capable of displaying a heterogenous polypeptide on its surface. Although one skilled in the art will appreciate that a variety of bacteriophage may be employed in the present invention, in preferred  
15 embodiments the vector is, or is derived from, a filamentous bacteriophage, such as, for example, f1, fd, Pfl, M13, *etc.* The filamentous phage may contain a selectable marker such as tetracycline (*e.g.*, "fd-tet"). Various filamentous phage display systems are well known to those of skill in the art (*see, e.g.*, Zacher *et al.* (1980) *Gene* 9: 127-140, Smith *et al.* (1985) *Science* 228: 1315-1317 (1985); and Parmley and Smith (1988) *Gene* 73: 305-318).

20 A "viral packaging signal" is a nucleic acid sequence necessary and sufficient to direct incorporation of a nucleic acid into a viral capsid.

An assembly cell is a cell in which a nucleic acid can be packaged into a viral coat protein (capsid). Assembly cells may be infected with one or more different virus particles (*e.g.* a normal or debilitated phage and a helper phage) that individually or in  
25 combination direct packaging of a nucleic acid into a viral capsid.

The term "detectable label" refers to any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical,  
30 biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads<sup>TM</sup>), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*,  $^3\text{H}$ ,  $^{125}\text{I}$ ,

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<sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (e.g., LacZ, CAT, horse radish peroxidase, alkaline phosphatase and others, commonly used as detectable enzymes, either as marker gene products or in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads. Those detectable labels that can be expressed by nucleic acids are referred to as "reporter genes" or "reporter gene products".

It will be recognized that fluorescent labels are not to be limited to single species organic molecules, but include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for example, CdSe-CdS core-shell nanocrystals enclosed in a silica shell can be easily derivatized for coupling to a biological molecule (Bruchez *et al.* (1998) *Science*, 281: 2013-2016). Similarly, highly fluorescent quantum dots (zinc sulfide-capped cadmium selenide) have been covalently coupled to biomolecules for use in ultrasensitive biological detection (Warren and Nie (1998) *Science*, 281: 2016-2018).

A nuclear localization signal is a nucleic acid sequence that directly or indirectly results in localization of the nucleic acid to the cell nucleus. Nuclear localization sequences (NLS) are well known to those of skill in the art. In most cases the NLS consists either of a short division of basic amino acids, for example as shown for the NLS of SV40 T antigen (PKKKRKV). Alternatively, the NLS may have a bipartite structure comprised of two stretches of basic residues separated by a spacer of about 10 amino acids. (Dingwell *et al.* (1991) *Trends Biochem. Sci.* 16: 478). In the practice of the invention, any NLS sequences that functions to direct the localization of PUR to the nucleus may be incorporated into the phage or phagemid vectors.

An endosomal escape sequence is a nucleic acid sequence that directly or indirectly results in the transport of a molecule from the endosome into the cytoplasm of a cell. Endosomal escape sequences (e.g. viral escape mechanisms) are well known to those of skill in the art. Examples include, but are not limited to the co-internalization system of adenovirus (Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, 8: 8850-8854), and the influenza viral peptides known to participate in endosomal escape mechanisms (Wiley and Skehel (1987) *Ann. Rev. Biochem.* 56: 365-394; Wagner *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, 89: 7934-7938).

The following abbreviations are used herein: AMP, ampicillin; c-erbB-2 ECD, extracellular domain of c-erbB-2; CDR, complementarity determining region; ELISA,

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enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FR, framework region; Glu, glucose; HBS, hepes buffered saline, 10 mM hepes, 150 mM NaCl, pH 7.4; IMAC, immobilized metal affinity chromatography;  $k_{on}$ , association rate constant;  $k_{off}$ , dissociation rate constant; MPBS, skimmed milk powder in PBS; MTPBS, skimmed milk powder in TPBS; PBS, phosphate buffered saline, 25 mM  $\text{NaH}_2\text{PO}_4$ , 125 mM NaCl, pH 7.0; PCR, polymerase chain reaction; RU, resonance units; scFv or scFv, single-chain Fv fragment; TPBS, 0.05% v/v Tween 20 in PBS; SPR, surface plasmon resonance;  $V_k$ , immunoglobulin kappa light chain variable region;  $V_\lambda$ , immunoglobulin lambda light chain variable region;  $V_L$ , immunoglobulin light chain variable region;  $V_H$ , immunoglobulin heavy chain variable region; wt, wild type.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the method for construction of a large human scFv phage antibody library. The strategy for library construction involved optimizing the individual steps of library construction to increase both the efficiency of scFv gene assembly and to increase the efficiency of cloning assembled scFv genes. (A). First, mRNA from lymphocytes was used to generate  $V_H$  and  $V_L$  gene repertoires by RTPCR which were cloned into different vectors to create  $V_H$  and  $V_L$  gene libraries of  $8.0 \times 10^8$  and  $7.2 \times 10^6$  members respectively. The cloned V-gene libraries provided a stable and limitless source of  $V_H$  and  $V_L$  genes for scFv assembly. DNA encoding the peptide  $(G_4S)_3$  was incorporated into the 5' end of the  $V_L$  library. This permitted generation of scFv genes by PCR splicing 2 DNA fragments. Previously, scFv gene repertoires were assembled from 3 separate DNA fragments consisting of  $V_H$ ,  $V_L$ , and linker DNA. (B)  $V_H$  and  $V_L$  gene repertoires were amplified from the separate libraries and assembled into an scFv gene repertoire using overlap extension PCR. The primers used to reamplify the  $V_H$  and  $V_L$  gene repertoires annealed 200 bp upstream of the 5' end of the  $V_H$  genes and 200 bp down stream of the  $V_L$  genes. These long overhangs ensured efficient restriction enzyme digestion. (C.) The scFv gene repertoire was digested with NcoI and NotI and cloned into the plasmid pHEN1 as fusions with the M13 gene III coat protein gene ( ) for phage-display.

Figures 2A 2B, and 2C show schematics illustrating antibody phage display: Cartoon of phage displaying (2A) a single scFv (2B) a single diabody or (2C) multiple scFv. scFv = single chain Fv antibody fragment;  $V_H$  = Ig heavy chain variable domain;  $V_L$  = Ig

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light chain variable domain; pIII = phage minor coat protein pIII; Ag = antigen bound by scFv.

Figure 3 shows the effect of trypsinization on the enrichment of antigen specific phage. A mixture of fd phage ( $5.0 \times 10^{11}$  cfu) and C6.5 scFv phagemid ( $5.0 \times 10^8$  fu) was incubated with SKBR3 cells for 2 hours at  $37^\circ\text{C}$ . Washes were performed either as described in Table 7 (-) or cells were trypsinized prior to cell lysis (+). Phage present in the first stripping buffer wash (cell surface phage) and the cell lysate (intracellular phage) were titrated in the presence of ampicillin (C6.5 phagemid) or tetracycline (fd phage).

Figure 4 shows the effect of incubation time and chloroquine on the recovery of antigen specific phage. SKBR3 cells were incubated in the presence (■, ●) or absence (□, ○) of chloroquine ( $50 \mu\text{M}$ ) for 2 hours prior to the addition of anti-botulinum phagemid (□, ■) or C6.5 scFv phagemid (○, ●) ( $1.5 \times 10^9$  cfu/ml). Cell samples were taken at 0 minutes, 20 minutes, 1 hour or 3 hours after phage addition, washed as described in Figure 4 including the trypsinization step and intracellular phages titrated.

Figure 5 shows the effect of phage concentration on the recovery of intracellular phage. Various concentrations of C6.5 scFv phagemid, C6ML3-9 scFv phagemid, C6.5 diabody phagemid or C6.5 scFv phage (input phage titer) were incubated with subconfluent SKBR3 cells grown in 6-well plates for 2 hours at  $37^\circ\text{C}$ . Cells were treated as described in Figure 4 including the trypsinization step and intracellular phage were titrated (output phage titer).

Figure 6 illustrates strategies for producing anti-ErbB2 phagemids and phages packaging a eukaryotic reporter gene. Left column: Helper phage are used to infect TG1 containing pHEN-F5-GFP, a phagemid composed of an f1 origin of replication (f1 ori), the anti-ErbB2 F5 scFv gene fused to gene III and an eukaryotic GFP reporter gene driven by the CMV promoter. Phage recovered from the culture supernatant display an average of 1 scFv-pIII fusion protein and 99% of them package the GFP reporter gene. Right column: the anti-ErbB2 F5 scFv gene is cloned into the fd phage genome for expression as a scFv-pIII fusion. fd-F5 phages are used to infect TG1 containing a GFP reporter phagemid vector (pcDNA3-GFP). Phages purified from the culture supernatant display multiple scFv-pIII fusion protein and approximately 50% package the GFP reporter gene.

Figure 7 shows a comparison of anti-ErbB2 phagemid and phage binding on cells.  $10^5$  ErbB2 expressing SKBR3 cells were incubated with increasing concentrations of



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F5-phagemids (circles) or fd-F5-phages (squares) at 4°C for 1 hour. Cell surface bound phages were detected with biotinylated anti-M13 and streptavidin-PE. Binding was detected by FACS and the results expressed as mean fluorescent intensity (MFI).

Figures 8A and 8B illustrate phagemid-mediated gene transfer in breast cancer cell lines. (Fig. 8A) (1, 2, 3)  $2.0 \times 10^5$  MCF7 (low ErbB2 expression) or (4, 5, 6)  $2.0 \times 10^5$  SKBR3 (high ErbB2 expression) cells grown in 6-well plates were incubated with either no (1,4) no phage, (2, 5)  $5.0 \times 10^{12}$  cfu/ml of helper phage packaging GFP or (3, 6)  $5.0 \times 10^{11}$  cfu/ml of F5-GFP-phagemids for 48 hrs. Cells were trypsinized and GFP detected by FACS. (Fig. 8B) An equal number of MCF7 and SKBR3 cells ( $1.0 \times 10^5$ ) were grown together and incubated with  $5.0 \times 10^{11}$  cfu/ml of F5-GFP-phagemids for 48 hrs. Cells were trypsinized and stained for ErbB2 expression using 4D5 antibody and rhodamine conjugated sheep anti-mouse Ig to discriminate SKBR3 (Region R1) and MCF7 (Region R2) cells. The GFP content of each subpopulation was determined by FACS.

Figures 9A, 9B, 9C, and 9D show concentration dependence and time course of phagemid mediated GFP expression in SKBR3 cells. Figures 9A and 9B show concentration dependence of phagemid and phage mediated GFP expression in SKBR3 cells.  $5.0 \times 10^4$  cells were grown in 24-well plates and incubated with increasing concentrations of F5-GFP-phagemid (squares), fd-F5-GFP-phage (diamonds) or GFP-helper phage (circles). After 60 hrs, the cells were trypsinized and analyzed by FACS for GFP expression. Figures 9C and 9D show the time dependence of phagemid mediated GFP expression in SKBR3 cells.  $5.0 \times 10^4$  cells were incubated with  $5.10^{11}$  cfu/mL of F5-GFP-phagemid and analyzed for GFP expression by FACS. For incubation times greater than 48 hrs, the phage were added to  $2.5 \times 10^4$  cells and the culture medium was replaced by fresh medium after 48 hrs of incubation. The results are expressed as (9A, 9C) % of GFP positive cells and (9B, 9D)) MFI of the GFP positive cells.

## DETAILED DESCRIPTION

### I. Transfection of cells using targeted phage.

This invention provides methods and materials for transfecting cells using targeted phage. In general the methods involve providing a phage displaying an external binding protein or antibody and containing a heterologous nucleic acid (heterologous to the

phage and/or to the cell). The external binding protein preferably specifically binds to an internalizing marker (receptor/receptor epitope) which results in internalization of the phage. Once internalized, the phage coat protein dissociates and the single stranded phage DNA genome is optionally expressed by the host cell machinery.

- 5 The antibody phage containing the heterologous nucleic acid can be prepared by a number of methods well known to those of skill in the art. In general these methods involve providing cells containing phagemid vector encoding the heterologous targeting protein or nucleic acid that is to be transfected into the cell and a corresponding page (e.g. helper phage) containing nucleic acid encoding a targeting polypeptide or the heterologous
- 10 nucleic acid that is to be transfected into the target cell. A bacterial (e.g. *E. coli*) cell is then infecting with the phage or phagemid or cotransfected with the phage or phagemid nucleic acids which are then repackaged into phage containing the desired nucleic acids. Several approaches are illustrated in Table 1.

15 Table 1. Strategies for the construction of phage nucleic acid delivery vehicles (vectors) of this invention.

E coli contains	Action	To Produce
Phagemid encoding Heterologous DNA; and DNA encoding scFv	<u>Infect</u> with helper phage.	Phage containing both heterologous nucleic acid sequences (targeting protein and heterologous DNA) and expressing targeting polypeptide (e.g. scFv) on surface.
Phagemid encoding heterologous DNA	<u>Infect</u> with helper phage containing nucleic acid encoding targeting polypeptide (scFv) on its surface	1) Phage containing targeting protein on surface and heterologous DNA (from phagemid clone); and 2) Phage containing targeting protein on surface and nucleic acid encoding targeting protein (from phage clone)
No phagemid or phage	<u>Co-transform</u> cell with phagemid and phage DNA and co-select, e.g., with antibiotics.	Phage containing both heterologous nucleic acid sequences (targeting protein and heterologous DNA) and expressing targeting polypeptide on surface.
No phagemid or phage	Infect cell with phage containing both heterologous DNA and DNA encoding targeting polypeptide.	Phage containing both heterologous nucleic acid sequences (targeting protein and heterologous DNA) and expressing targeting polypeptide on surface.

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No phagemid or phage	Infect cell with phage containing heterologous DNA and phagemid containing targeting polypeptide (e.g., scFv).	1) Phage containing targeting polypeptide and heterologous DNA; and 2) Phage containing only heterologous DNA.
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\*phagemid contains packaging signal.

In a preferred embodiment, phage expressing the targeting molecule on their surface are used as helper phage (see Maniatis) to package the genome of a phagemid containing the heterologous DNA (e.g. a mammalian expression cassette).

In one preferred embodiment, this involves subcloning the targeting protein gene from the pHEN1 vector into a phage vector (such as FdDOG1 (Clackson *et al*, *Nature* (1991) 352: 624-628) where it is located inframe with the phage gene III. For example, targeting scFv can be cloned as ApaI-NotI fragments into the ApaI-NotI sites of FdDOG1. The phage genome leads to production of phage (from bacteria) which has the targeting protein on its surface and the phage genome inside. These phage are then used for superinfection of the phagemid containing bacterial cells. The phagemid vector DNA by definition contains a phage origin of replication and packaging signal. As a result, the phage genome products direct single stranded DNA synthesis of the phagemid DNA. The phage acts as a helper phage leading to the production of two types of phage particles, those that contain the phage genome and those that contain the phagemid genome. Using standard phage (such as Fd) as helper phage results in approximately an equal probability of the phage packaging either genome. All of the phage will also have the targeting protein on their surface as pIII fusions. This is a simple way to generate phage that have the targeting molecule on their surface and the heterologous expression DNA inside the phage. While only a fraction (50%) of phage harbor the heterologous expression DNA, this is a large enough fraction given the high titer with which phage can be produced, to generate targeted phage.

Packaging is rapid, simple and most importantly can avoid tedious and time consuming subcloning steps required to insert the DNA sequence that is to be delivered to the eukaryotic cells into the phagemid or phage vector harboring the DNA sequence of the targeting gene. Thus this approach provides a generic method for packaging any DNA into the targeting phage for delivery and expression in eukaryotic cells. This makes it simple to deliver and study the effects of a large number of different genes in eukaryotic cells.

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It is noted that use of phage genome as a helper phage can lead to the problem of "interference" where the titer of phage generated is lower than expected (see Maniatis). It is also known to those skilled in the art, that a number of phage vectors exist which can overcome this problem. One of these, helper phage K07, uses a phage with a plasmid origin of replication and a partially disabled phage origin of replication (see *Maniatis*). Use of K07 as a helper phage leads to production of higher phage titers. Thus similar alternative phage vector backbones could be used for creation of targeted helper phage to result in higher phage titers.

In a variant of this embodiment, the bacteria can be co-transformed with phage and phagemid DNA and co-selected with antibiotics. The resulting cells contain both genomes and make phage containing both the heterologous targeting protein and the nucleic acid that is to be delivered into the cell.

In still another embodiment, the phage contains the heterologous nucleic acid that is to be delivered into the cell and the phagemid contains the nucleic acid encoding the heterologous targeting protein.

In yet another embodiment, the phagemid genome containing the targeting molecule-pIII gene fusion is modified to contain the gene sequence that is to be delivered to the target eukaryotic cell (for example a mammalian expression cassette containing a reporter gene (or cDNA) and/or another gene or (cDNA)). Targeting phage are produced in the standard manner by the addition of helper phage.

In still yet another embodiment, both the heterologous nucleic acid that is to be delivered into the cell and the heterologous nucleic acid encoding the targeting protein (e.g. single-chain antibody) are inserted into the phage genome. The bacteria then need only the phage genome inside and will make phage with targeting protein on the outside and both genomes inside.

While it is demonstrated herein that targeted phage can be delivered via an internalizing receptor into the endosome, it is recognized that other factors may reduce the efficiency of gene expression. The phage preferably from the endosome and uncoating facilitates exposure of the single stranded genome which then finds its way to the nucleus. There the single stranded DNA is replicated to double stranded DNA which is then transcribed and translated. It is also recognized by those skilled in the art, that methods exist to improve the efficiency of each of these steps. For example, endosomal escape sequences

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are known which can be incorporated into the phage coat proteins. Co incubation with defective adenovirus would also provide endosome escape signals. Nuclear localization sequences are also known which could increase delivery to the nucleus. Inclusion of episomal replication sequences lead to amplification of the delivered DNA with an increase in the efficiency of expression.

## II. Target cells.

Virtually any cell bearing an internalizing marker/receptor can be transfected using the methods of this invention. Using the assays described above and illustrated in the examples, internalizing phage display library members can be optimized for internalization by a particular marker. Alternatively or in addition, new, previously unknown receptors or epitopes can be identified and targeted.

Targets can be selected that whose distribution is restricted to particular cell types, target tissues, organs, or cells and/or tissues and/or organs displaying a particular physiological state or pathological condition. Thus, for example, targets can be selected that are characteristic of particular tumor types. Tumor specific targets are well known to those of skill in the art and include, but are not limited to c-erbB-2, the IL-13 receptor, other growth factor receptors, and so forth.

Alternatively, internalizing targets can be selected that are present on most or all cell types (e.g., transferrin receptor). Also, it is possible to select a phage library to identify such targeting molecules. For example, a scFv phage library can be selected without a subtracting cell line, or sequentially on unrelated cell lines. We in fact have already identified scFv using this approach that bind to all cell types tested. In this instance the transfection methods allow generalized transfection of essentially any and/or all cells or an organ, tissue, or organism.

Tissue specific targets can also be identified. Thus, for example, it is noted that Ruoslahti *et al.* (U.S. Patent No: 5,622,699 have identified polypeptides that specifically target particular tissues (e.g. brain, kidney, etc.).

## III. Transfected nucleic acids.

Using the above-described methods, virtually any heterologous nucleic acid can be transfected into a cell. Once in the cell, the nucleic acid will optionally be transcribed, and optionally translated, depending on the nature of the particular nucleic acids.

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In one embodiment, the heterologous nucleic acid can encode a polypeptide gene product it is desired to introduce into the cell. Such a polypeptide gene product may include a reporter gene (e.g., green fluorescent protein or  $\beta$ -galactosidase). For killing a cell (such as a tumor cell) one might deliver the TK gene or the gene encoding a toxin (such as *Pseudomonas*

5 exotoxin or subunits thereof, diphtheria toxin or subunits thereof, ricin, abrin, etc.). Alternatively, the nucleic acid transcript can be active in its own right (e.g. a ribozyme, an antisense molecule, etc.).

Where a heterologous nucleic acid encodes a protein product that is to be expressed in the target cell, the heterologous nucleic acid preferably encodes an expression cassette compatible with the target cell. Thus, for example, where the target cell is a mammalian cell, the expression cassette preferably includes a promoter that is inducible or constitutive in a mammalian cell, an initiation site, and a termination site. The cassette can optionally include a selectable marker.

#### IV. Identifying internalizing antibodies and/or targets (e.g. receptors).

##### A) Identification of internalizing polypeptides/antibodies.

15 The transduction methods of this invention rely on the use of "internalizing antibodies", or "internalizing polypeptides". Such "internalizing" molecules are internalized when they bind a target cell. Methods of identifying internalizing antibodies/target epitopes are provided herein and illustrated in the Examples. The methods generally involve contacting a "target" cell with one or more members of a phage display library displaying an antibody or a binding polypeptide. The phage display library is preferably a polyvalent phage display library and it is believed that this invention provides the first description of a polyvalent antibody phage display library.

25 After a suitable incubation period, the cells are washed to remove externally bound phage (library members) and then internalized phage are released from the cells, e.g., by cell lysis. It was a discovery of this invention that the internalized phage are still viable (infectious). Thus the internalized phage in the cell lysate can be recovered and expanded by using the lysate containing internalized phage to infect a bacterial host. Growth of infected bacteria leads to expansion of the phage which can be used for a subsequent round of selection. Each round of selection enriches for phage which are more efficiently internalized, more specific for the target cell or have improved binding characteristics.

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The phage display library is preferably contacted with a subtractive cell line-- (i.e. a subtractive cell line is added to the target cells and culture media) to remove members of the phage display library that are not specific to the "target" cell(s). The subtractive cell line is preferably added under conditions in which members of the phage display library are not internalized (e.g., at a temperature of about 4°C to about 20°C, more preferably at a temperature of about 4°C) so that non-specific binding members of the library are not internalized (sequestered) before they can be subtracted out by the subtractive cell line.

After subtracting out non-specific binding antibodies, the "target" cells are washed to remove the subtractive cell line and to remove non-specifically or weakly-bound phage."

The target cells are then cultured under conditions where it is possible for internalization to occur (e.g. at a temperature of about 35°C to about 39°C, more preferably at a temperature of about 37°C). The duration of the internalization culture period will determine the internalization speed of the antibodies (phage display members) for which selection takes place. With shorter internalization periods more rapid internalizing antibodies are selected while with longer internalization periods slower internalizing antibodies are selected. The internalization period is preferably less than about 120 minutes, more preferably less than about 60 minutes, and most preferably less than about 30 minutes or even less than about 20 minutes.

It is noted that during the internalization period the target cells are grown under conditions in which internalization can occur. For a number of cell lines, this involves culturing the cells adherently on culture plates.

After internalization has been allowed to occur the target cells are washed to remove non-internalized (e.g. surface-bound phage).

The cells can then be moved to clean media. In a preferred embodiment, where the cells are adherent, they cells are trypsinized to free the cells from the extracellular matrix which may contain phage antibodies that bind the extracellular matrix. Freeing the cells into solution permits more through washing and moving of the cells to a new culture flask will leave behind any phage that may have stuck to the tissue culture dish.

The cells can then be washed with a large volume of PBS and lysed to release the internalized phage which can then be expanded e.g. used to infect *E. coli* to produce phage for the next round of selection. It is noted that there is no need to actually visualize

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the internalized phage. Simple cell lysis and expansion of the formerly internalized phage is sufficient for recovering internalizing phage display members.

**B) Identification of internalizing receptors.**

Once an antibody or polypeptide that is internalized into a cell has been identified, it is possible to probe one or more cell types with the identified antibody or polypeptide to identify the target recognized and bound by the antibody. Since the antibody is an internalizing antibody it is likely that such targets are themselves internalizing targets (e.g. members or portions of internalizing receptors).

In one embodiment, the antibody can be labeled as described below. The cells can then be contacted with the antibody (i.e. *in vivo* or *in vitro*) and the cells or cellular regions to which the antibody binds can then be isolated.

Alternatively, the antibodies can be used e.g. in an affinity matrix (e.g. affinity column) to isolate the targets (e.g. receptor or receptor subunits) to which they bind. Briefly, in one embodiment, affinity chromatography involves immobilizing (e.g. on a solid support) one or more species of the internalizing antibodies identified according to the methods of this invention. Cells, cellular lysate, or cellular homogenate are then contacted with the immobilized antibody which then binds to its cognate ligand. The remaining material is then washed away and the bound/isolated cognate ligand can then be released from the antibody for further use. Methods of performing affinity chromatography are well known to those of skill in the art (see, e.g., U.S. Patent Nos: 5,710,254, 5,491,096, 5,278,061, 5,110,907, 4,985,144, 4,385,991, 3,983,001, etc.).

In another embodiment, the antibodies are used to immunoprecipitate the target from cell lysate. The precipitate is then run on an SDS-PAGE gel which is Western blotted onto nitrocellulose. The blot is probed with the precipitating antibody to identify the location of the target. The portion of the blot containing the target can then be sent for N-terminal protein sequencing. The N-terminal sequence can then be used to identify the target from standard databases, or DNA probes can be synthesized to probe genomic or cDNA libraries. This approach has been used to identify the antigen bound by a phage antibody. Selections of a phage antibody library were done on intact *Chlamydia trachomatis* (a bacterial like organism that causes Chlamydial diseases). Selected antibodies were then used as described above to identify the antigen bound.



### C) Assay Components

#### 1) Phage display library.

##### a) Mono-valent antibody libraries and polypeptide libraries.

The ability to express polypeptide and antibody fragments on the surface of viruses which infect bacteria (bacteriophage or phage) makes it possible to isolate a single binding polypeptide or antibody fragment from a library of greater than  $10^{10}$  nonbinding clones. To express polypeptide or antibody fragments on the surface of phage (phage display), a polypeptide or an antibody fragment gene is inserted into the gene encoding a phage surface protein (pIII) and the antibody fragment-pIII fusion protein is displayed on the phage surface (McCafferty *et al.* (1990) *Nature*, 348: 552-554; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137). Since the antibody fragments on the surface of the phage are functional, phage bearing antigen binding polypeptides or antibody fragments can be separated from non-binding phage by antigen affinity chromatography (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Depending on the affinity of the antibody fragment, enrichment factors of 20 fold - 1,000,000 fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, however, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round can become 1,000,000 fold in two rounds of selection (McCafferty *et al.* (1990) *Nature*, 348: 552-554). Thus even when enrichments are low (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597), multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results in enrichment, the majority of clones bind antigen after four rounds of selection. Thus only a relatively small number of clones (several hundred) need to be analyzed for binding to antigen.

In a preferred embodiment, analysis for binding is simplified by including an amber codon between the antibody fragment gene and gene III. The amber codon makes it possible to easily switch between displayed and soluble (native) antibody fragment simply by changing the host bacterial strain (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137).

Human antibodies can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597). In the first Example, natural  $V_H$  and  $V_L$  repertoires present in human peripheral

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blood lymphocytes were isolated from unimmunized donors by PCR. The V-gene repertoires were spliced together at random using PCR to create a scFv gene repertoire which was cloned into a phage vector to create a library of 30 million phage antibodies (*Id.*). From this single "naive" phage antibody library, binding antibody fragments have been isolated against more than 17 different antigens, including haptens, polysaccharides and proteins (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; Marks *et al.* (1993) *Bio/Technology*. 10: 779-783; Griffiths *et al.* (1993) *EMBO J.* 12: 725-734; Clackson *et al.* (1991) *Nature*. 352: 624-628). Antibodies have been produced against self proteins, including human thyroglobulin, immunoglobulin, tumor necrosis factor and CEA (Griffiths *et al.* (1993) *EMBO J.* 12: 725-734). It is also possible to isolate antibodies against cell surface antigens by selecting directly on intact cells. For example, antibody fragments against four different erythrocyte cell surface antigens were produced by selecting directly on erythrocytes (Marks *et al.* (1993) *Bio/Technology*. 10: 779-783). Antibodies were produced against blood group antigens with surface densities as low as 5,000 sites/cell. The antibody fragments were highly specific to the antigen used for selection, and were functional in agglutination and immunofluorescence assays. Antibodies against the lower density antigens were produced by first selecting the phage antibody library on a highly related cell type which lacked the antigen of interest. This negative selection removed binders against the higher density antigens and subsequent selection of the depleted phage antibody library on cells expressing the antigen of interest resulted in isolation of antibodies against that antigen. With a library of this size and diversity, at least one to several binders can be isolated against a protein antigen 70% of the time. The antibody fragments are highly specific for the antigen used for selection and have affinities in the 1 :M to 100 nM range (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; Griffiths *et al.* (1993) *EMBO J.* 12: 725-734). Larger phage antibody libraries result in the isolation of more antibodies of higher binding affinity to a greater proportion of antigens.

The creation of a suitable large phage display antibody library is described in detail in Example 1.

#### b) Polyvalent antibody phage display libraries

The probability of selecting internalizing antibodies from a phage-display antibody library is increased by increasing the valency of the displayed antibody. This approach takes advantage of normal cell-surface receptor biology. Often cell-surface

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receptors (e.g. growth factor receptors) activate upon binding their cognate ligand through a process of homo- or heterodimerization (or trimerization, or tetramerization, etc.). The association of the receptor subunits in this process can be mediated directly (e.g. when bound by a bivalent ligand) or indirectly by causing a conformational change in the receptor.

5           It was a discovery of this invention that polyvalent antibodies in a display library (e.g. a phage display library) can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cytosol. Thus, to increase the likelihood of identifying internalizing antibodies or recognizing internalizing epitopes, preferred embodiments of this invention utilize a polyvalent phage display antibody library. It is  
10           believed that no multivalent phage-display antibody libraries have been created prior to this invention. Unlike the multivalently displayed peptide phage libraries, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95: 6157-  
15           6162.).

          As used herein, a polyvalent phage display antibody library, refers to a library in which each member (e.g. phage particle) displays, on average) two or more binding domains, wherein each binding domain includes a variable heavy and a variable light region. More generally, a multivalent phage display library displays, on average, two or more pIII  
20           fusions per phage particle. Polyvalent phage display can be achieved by expressing diabodies (i.e., a protein formed by fusion or conjugation of two single chain antibodies (e.g. scFv)) or by display of, on average, two or more antibodies on each phage particle. In contrast, a mono-valent library displays, on average, one single-chain antibody per viral particle.

#### i) Diabody expression.

25           Diabodies are scFv dimers where each chain consists of heavy ( $V_H$ ) and light ( $V_L$ ) chain variable domains connected using a linker (e.g. a peptide linker) that is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (Holliger *et al.* (1993) *Proc. Natl. Acad. Sci.* 90: 6444-6448).  
30           The C6.5 diabody was constructed by shortening the peptide linker between the Ig  $V_H$  and  $V_L$  domains from 15 to 5 amino acids and binds ErbB2 on SKBR3 cells bivalently with a  $K_d$

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approximately 40 fold lower than C6.5 ( $4.0 \times 10^{-10}$  M) (Adams *et al.* (1998) *Brit. J. Cancer*. 77: 1405-1412, 1998).

In Example 5, described herein, C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137). This yielded phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (Marks *et al.* (1992) *J. Biol. Chem.* 267: 16007-16010). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule. Using the teachings provided herein one of skill in the art can routinely produce other  
10 diabodies.

Phage displaying bivalent diabodies or multiple copies of scFv were more efficiently endocytosed than phage displaying monomeric scFv and recovery of infectious phage was increased by preincubation of cells with chloroquine.

The results indicate that it is possible to select for endocytosable antibodies,  
15 even at the low concentrations that would exist for a single phage antibody member in a library of  $10^9$  members.

#### ii) Polyvalent display of single-chain antibodies.

As an alternative to the use of diabodies, antibody phage display libraries are created in which each viral particle, on average, expresses at least 2, preferably at least 3,  
20 more preferably at least 4, and most preferably at least 5 copies of a single chain antibody.

In principle, each copy of pIII on the page (and there is controversy as to whether there are 3 or 5 copies of pIII per phage) should express an antibody. However, proteolysis occurs and the number actually displayed is typically less. Thus, preferred multivalent antibody libraries are constructed in a phage vector and not a phage mid vector.  
25 This means that helper phage need not be added to make phage. Helper phage bring into the *E. coli* wild-type pIII that competes with the scFv-pIII fusion. Thus, in phagemid vector, this competition leads, on average, to only 1 (one less) antibody per phage.

To produce multivalent antibody libraries, the single chain antibodies, typically expressed in phagemid, are subcloned from the phagemid vector into a phage  
30 vector. No helper phage is required and there is no competition between the wild-type pIII and the fusion scFv pIII fusion. thus, on average, the phage display two or more pIII fusions. Thus, by way of illustration, Example 5 describes the subcloning of the C6.5 scFv

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gene into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein. Other phage vectors suitable for such use are well known to those of skill in the art.

## 2) Target cells.

5           The target cells of this invention include any cell for which it is desired to identify an internalizing polypeptide or antibody or for which it is desired to identify an internalizing marker (e.g. receptor). The cells can include cells of multicellular eukaryotes, uni-cellular eukaryotes, including plants and fungi, and even prokaryotic cells. Preferred target cells are eukaryotic, more preferably vertebrate cells, and most preferably mammalian  
10   cells (e.g. cells from murines, bovines, primates including humans, largomorphs, canines, felines, and so forth). The cells can be normal healthy cells or cells characterized by a particular pathology (e.g. tumor cells).

          Target cells can include any cell type where it would be useful to: 1) have an antibody specifically recognize the cell type or related cell types (for example for cell  
15   sorting, cell staining or other diagnostic procedures); 2) have a ligand which is specifically internalized into the cell type or related cell types (for example to deliver a toxic or therapeutic gene or protein). Additional target cells include, but are not limited to differentiated cells (i.e. differentiated to become a tissue, e.g. prostate, breast). Thus an antibody that recognized and killed prostate cells would be good for prostate cancer even if it  
20   killed normal prostate cells (the prostate is not an essential organ). Target cells may include tissue specific cells, and cells at a given developmental stage. Target cells may also include precursor cells, e.g. bone marrow stem cells, would be useful for isolating, perhaps stimulating for differentiation.

          Target cells can also include cell lines transfected with a gene for a known  
25   receptor (for example ErbB2) to which it would be useful to have internalizing antibodies.

          Many ErbB2 antibodies are not internalizing. Rather than immunizing with recombinant protein or selecting a phage library on recombinant protein, selection on ErbB2 transfected cells for internalization should yield precisely antibodies with the desired characteristics (internalization). Finally, a cDNA library could be transfected into a cell line  
30   (for example COS) from a desired target cell line or tissue and phage antibodies selected for internalization. After several rounds of selection, the phage could be used to stain and sort

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(for example by FACS) transfected cells. DNA can be recovered from the cells, yielding the sequences of internalizing receptors as well as phage antibodies that bind them.

### 3) Cells of a subtractive cell line.

In a preferred embodiment of the assays of this invention, the phage display library is contacted with cells from a "subtractive" cell line. This step is intended to deplete or eliminate members of the phage display library that either bind the cells non-specifically or that bind to targets other than the target against which it is desired to obtain a binding polypeptide or antibody. The contacting with the cells from a "subtractive" cell line can occur before, during, or after the target cells are contacted with members of the phage display library. However, in a preferred embodiment, the contacting with cells of a subtractive cell line is simultaneous with contacting of the target cells. Thus, for example, in a preferred embodiment the target cell line (grown adherent to a tissue culture plate) is co-incubated with the subtracting cell line (in suspension) in a single cell culture flask.

Virtually any cell can act as a subtractive cell. However, in a preferred embodiment, subtractive cells display all the markers on the target cell except the marker (e.g. receptor) that is to act as a target for selection of the desired binding antibodies or binding polypeptides. Particularly preferred cells are thus closely related to the target cell(s), in terms of having common internalizing cell surface receptors (such as transferrin); for example fibroblasts. If one was selecting on a tumor cell line (for example a breast tumor cell line), then one could negatively select on a normal breast cell line. This may, however, deplete for antibodies that bind to overexpressed antigens, so again a parallel path would be to negatively select on fibroblasts. If one was using transfected cells, then non-transfected cell could be used as the subtractive cell line. Where the tumor is epithelial in origin, the preferred subtractive cell will also be epithelial and even more preferably from the same tissue or organ.

Particularly preferred subtractive cells include, but are not limited to, non-differentiated cell lines, non-transfected cells, mixtures of non-differentiated and non-transfected cells. When selecting for internalization on tumor cells, preferred subtractive cell lines are preferably the non-tumor cells of the same tissue (for example, breast tumor cells versus normal breast epithelial cells). Also, for cDNA expression libraries, the subtractive cell line will be the non-transformed cell line used for library construction (e.g. COS, CHO, etc.).

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In one particularly preferred embodiment, the "target" cell is a cell transformed with a gene or cDNA for a specific target receptor. In this instance, the subtractive cell line is preferably the non-transformed cell line. Thus for example where CHO cells are transformed with a vector containing the gene for the EGF receptor, the EGF-expressing cells are used as the target cell line, and the subtractive cell line is the untransformed CHO cells. Using this approach internalizing anti-EGF receptor antibodies were obtained.

The subtractive cells are more effective when provided in excess over the target cells. The excess is preferably at least about a 2-fold to about a 1000-fold excess, more preferably about a 3-fold to about a 100-fold excess, and most preferably about a 5-fold to about a 50-fold excess. In one embodiment, a 5-fold excess is sufficient.

#### 4) Washing steps.

As indicated above a variety of washing steps are used in the methods of this invention. In particular, a "weak" washing step can be used to remove the subtractive cells and weakly or non-specifically binding members of the phage display library. A second strong washing step is preferably used after internalization of members of the phage display library. The "strong" washing step is intended to remove tightly- and weakly-bound surface phage.

Buffers and methods for performing weak and strong wash steps are well known to those of skill in the art. For example, weak washes can be done with standard buffers or culture media (e.g., phosphate buffered saline (buffer) DMEM (culture media), etc.).

#### 5) Culturing under internalizing conditions.

As explained above, the cells are preferably cultured under "internalizing" conditions. Internalizing culture conditions are conditions in which the cell when bound by a member of a phage display library at an appropriate (e.g. internalizing) site or receptor, transports the bound member into the cell. This can involve transport into a vesicle, into the endoplasmic reticulum, the golgi complex, or into the cytosol of the cell itself.

Internalizing conditions are most easily achieved when the cells are cultured under conditions that mimic those of the cell in its native state. Thus many cells, e.g. epidermal cells, preferably grow as adherent layers attached to a basement membrane. Such

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cells more effectively internalize binding polypeptides and antibodies when they are cultured as adherent monolayers. Chloroquine and serum free medium both avoid non specific internalization and enhance specific internalization (ligands in the serum that induce the internalization of receptor of interest and take with them non specific phages being in the neighborhood). In addition, for internalization to occur, the cells should be cultured at a temperature and pH that permits internalization. Suitable temperature and pH range from about 35°C to about 39°C and from pH 6 to about pH 8, more preferably from about pH 6.5 to about pH 7.5, with preferred temperature and pH being about 37°C and pH 7.5 respectively. In a preferred embodiment, the cells are preincubated in serum culture medium for about two hours before adding the phages and the competitor (subtraction) cells.

#### 6) Identification of internalized phage

The internalized phage display library members can be identified directly or indirectly. Direct identification can be accomplished simply by visualizing the phage within a cell *e.g.* via immunofluorescent or confocal microscopy. Phage internalization can be identified by their ability to deliver a reporter gene that is expressed within the cell. The reporter gene can be one that produces a detectable signal (*e.g.* a fluorescent (*e.g.* lux, green fluorescent protein, *etc.*) or colorimetric signal (*e.g.* HRP,  $\beta$ -galactosidase) or can itself be a selectable marker (*e.g.* an antibiotic resistance gene). The use of both  $\beta$ -galactosidase and GFP as reporter genes in such phage is described herein.

Alternatively, the phage display member can bear a marker (*e.g.* a label) and cells containing the internalized phage can be detected simply by detection of the label (*e.g.* in a flow cytometer). The direct methods preferably used for identification of the receptors or cells that are bound after selections are performed. It is noted that cell sorting approaches (FACs) will work with identification of either surface bound or internalized phage.

However, an additional level of specificity can be achieved if the cells are first sorted for the presence of internalized phage prior to lysis. Direct methods are also used during the analysis phase to demonstrate that the phage selected are indeed internalized.

Alternatively the internalized phage display library members can be identified indirectly. In indirect detection methods the phage-display library member(s) do not need to be detected while they are present within the cell. It is sufficient that they simply have been internalized.



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Indirect identification is accomplished for example, by isolating and expanding the phage that were internalized into the cells as described below. Indirect identification is particularly well suited where the identified phage display library members are going to be used in subsequent rounds of selection or to isolate bacteria harboring monoclonal phage genomes for subsequent monoclonal phage characterization (that is for the analysis of selection results).

#### 7) Isolation and expansion of internalized phage.

It was a discovery of this invention that phage display library members that have been internalized into target cells (e.g. mammalian tumor cells) remain viable and can be recovered and expanded into a "selected" library suitable for subsequent rounds of selection and/or isolation and characterization of particular members.

As used herein, the term "recovery" is intended to include recovery of the infectious phage and/or recovery of the phage antibody gene and/or recovery of a heterologous nucleic acid accompanying the antibody gene.

The internalized phage can be isolated and expanded using standard methods. Typically these include lysing the cells (e.g., with 100 mM triethylamine (high pH)), and using the lysate to infect a suitable bacterial host, e.g., *E. coli* TG1. The phage-containing bacteria are then cultured according to standard methods (see, e.g., Sambrook *supra*, Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597).

#### V. Libraries and vectors.

In another embodiment, this invention provides libraries and vectors for practice of the methods described herein. The libraries are preferably polyvalent libraries, including diabody libraries and more preferably including multi-valent single chain antibody libraries (e.g. scFv), (e.g., expressed by phage).

The libraries can take a number of forms. Thus, in one embodiment the library is a collection of cells containing members of the phage display library, while in another embodiment, the library consists of a collection of isolated phage, and in still library consists of a library of nucleic acids encoding a polyvalent phage display library. The nucleic acids can be phagemid vectors encoding the antibodies and ready for subcloning into a phage vector or the nucleic acids can be a collection of phagemid already carrying the subcloned antibody-encoding nucleic acids.

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Other preferred vectors include the phage itself carrying expressing a heterologous binding domain (e.g. an antibody) and containing a heterologous nucleic acid that is to be delivered into the target cell(s). While in some embodiments, the heterologous nucleic acid expresses a detectable label or is itself a label (e.g. a unique sequence detectable by hybridization or amplification (e.g. PCR) methods) in other embodiments, the heterologous nucleic acid includes a nucleic acid that encodes a molecule other than a detectable label (e.g. a polypeptide, an antisense molecule, a ribozyme, etc.).

#### VI. Transformation of cells.

This invention provides new methods for effective transfection of cells both *in vivo* and *ex vivo* (*in vitro*). Virtually any cell, eukaryotic or prokaryotic, can be transfected according to the methods of this invention. Particularly preferred cells are eukaryotic cells, more preferably vertebrate (e.g., mammalian) cells. Other cells, however, can also be transfected. Such cells include, but are not limited to bacterial cells (e.g. bacteria not typically infected by phage), fungal or yeast cells (e.g. to deliver a cytotoxin in the treatment of fungal or yeast infections), algal cells, insect cells, and the like.

A virtually limitless variety of nucleic acids can be transfected into "target cells". The nucleic acids can be selected to express particular polypeptide(s), or the nucleic acids can have an activity themselves (e.g. antisense molecules, ribozymes). Such expressed heterologous genes (or cDNAs), antisense molecules, or ribozymes are useful in a wide variety of applications and, for example, have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts.

The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies. As an example, *in vivo* expression of cholesterol-regulating genes, genes which selectively block the replication of HIV, and tumor-suppressing genes in human patients dramatically improves the treatment of heart disease, AIDS, and cancer, respectively. For a review of gene therapy procedures, see Anderson (1992) *Science* 256:808-813; Nabel and Felgner (1993) *TIBTECH* 11: 211-217; Mitani and Caskey (1993) *TIBTECH* 11: 162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) *TIBTECH* 11: 167-175; Miller (1992) *Nature* 357: 455-460; Van Brunt (1988) *Biotechnology* 6(10): 1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada *et al.*

(1995) in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu *et al.* (1994) *Gene Therapy* 1: 13-26.

Delivery of the gene or genetic material into the cell is the first critical step in gene therapy treatment of disease, in a wide variety of research systems, in the development of knockout (KO) mice, in the development and modification of cell lines, and the like. It will be appreciated that the transfection methods of this invention greatly facilitate the delivery of nucleic acids into cells in these and other contexts.

A) Ex vivo transformation.

For example, *ex vivo* cell transformation for diagnostics, research, or for gene therapy (*e.g.*, via re-infusion of the transformed cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected a heterologous gene according to the methods of this invention, and re-infused back into the subject organism (*e.g.*, patient). Various cell types suitable for *ex vivo* transformation are well known to those of skill in the art. Particular preferred cells are progenitor or stem cells (*see, e.g.*, Freshney *et al.* (1994) *Culture of Animal Cells, a Manual of Basic Technique*, third edition Wiley-Liss, New York) and the references cited therein for a discussion of how to isolate and culture cells from patients).

In one particularly preferred embodiment, stem cells are used in *ex-vivo* procedures for cell transformation and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such as GM-CSF, IFN- $\gamma$  and TNF- $\alpha$  are known (*see, Inaba et al.* (1992) *J. Exp. Med.* 176: 1693-1702, and Szabolcs *et al.* (1995) 154: 5851-5861).

Stem cells are isolated for transduction and differentiation using known methods. For example, in mice, bone marrow cells are isolated by sacrificing the mouse and cutting the leg bones with a pair of scissors. Stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells). For an example of this protocol *see, Inaba et al.* (1992) *J. Exp. Med.* 176: 1693-1702.

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In humans, bone marrow aspirations from iliac crests are performed e.g., under general anesthesia in the operating room. The bone marrow aspiration is approximately 1,000 ml in quantity and is collected from the posterior iliac bones and crests. If the total number of cells collected is less than about  $2 \times 10^8/\text{kg}$ , a second aspiration using the sternum and anterior iliac crests in addition to posterior crests is performed. During the operation, two units of irradiated packed red cells are administered to replace the volume of marrow taken by the aspiration. Human hematopoietic progenitor and stem cells are characterized by the presence of a CD34 surface membrane antigen. This antigen is used for purification, e.g., on affinity columns which bind CD34. After the bone marrow is harvested, the mononuclear cells are separated from the other components by means of ficoll gradient centrifugation. This is performed by a semi-automated method using a cell separator (e.g., a Baxter Fenwal CS3000+ or Terumo machine). The light density cells, composed mostly of mononuclear cells are collected and the cells are incubated in plastic flasks at 37°C for 1.5 hours. The adherent cells (monocytes, macrophages and B-Cells) are discarded. The non-adherent cells are then collected and incubated with a monoclonal anti-CD34 antibody (e.g., the murine antibody 9C5) at 40°C for 30 minutes with gentle rotation. The final concentration for the anti-CD34 antibody is 10  $\mu\text{g}/\text{ml}$ . After two washes, paramagnetic microspheres (DynaBeads, supplied by Baxter Immunotherapy Group, Santa Ana, California) coated with sheep antimouse IgG (Fc) antibody are added to the cell suspension at a ratio of 2 cells/bead. After a further incubation period of 30 minutes at 40°C, the rosetted cells with magnetic beads are collected with a magnet. Chymopapain (supplied by Baxter Immunotherapy Group, Santa Ana, California) at a final concentration of 200 U/ml is added to release the beads from the CD34+ cells. Alternatively, and preferably, an affinity column isolation procedure can be used which binds to CD34, or to antibodies bound to CD34 (see, the examples below). See, Ho et al. (1995) *Stem Cells* 13 (suppl. 3): 100-105. See also, Brenner (1993) *Journal of Hematotherapy* 2: 7-17.

In another embodiment, hematopoietic stem cells are isolated from fetal cord blood. Yu et al. (1995) *Proc. Natl. Acad. Sci. USA* 92: 699-703 describe a preferred method of transducing CD34+ cells from human fetal cord blood using retroviral vectors.

#### B) In vivo transformation.

The vectors of this invention (phage expressing a specific targeting antibody and containing a heterologous nucleic acid in an expression cassette) can be administered

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directly to the organism for transduction of cells *in vivo*. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. The phage packaged nucleic acids are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such packaged nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention.

#### VII. Formulations for transformation of cells.

As indicated above, particular when administered *in vivo*, the vectors of this invention (targeted phage containing heterologous nucleic acid(s)) are compounded in a formulation in combination with a pharmaceutically acceptable excipient (*i.e.*, a pharmaceutical formulation). Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the vector(s) of this invention suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The vectors of this invention, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be

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administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable  
5 suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged nucleic acid with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by  
10 intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile  
15 suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of packaged nucleic acid can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

20 Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by vectors of this invention as described above in the context of *ex vivo* therapy can also be administered intravenously or parenterally as described above.

The dose administered to a patient, in the context of the present invention  
25 should be sufficient to effect detectable transformation, more preferably sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany  
30 the administration of a particular vector, or transduced cell type in a particular patient.

In determining the effective amount of the vector to be administered in the, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression

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of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 µg to 1 g for a typical 70 kilogram patient, and doses of vectors which include a phage particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

5 For administration, inhibitors and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

10 In a preferred embodiment, prior to infusion, blood samples are obtained and saved for analysis. Between  $1 \times 10^8$  and  $1 \times 10^{12}$  transduced cells are infused intravenously over 60-200 minutes. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for subsequent analysis. Leukopheresis, transduction and reinfusion can be repeated are  
15 repeated every 2 to 3 months. After the first treatment, infusions can be performed on a outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4, and preferably 8 hours following the therapy.

Transduced cells are prepared for reinfusion according to established methods. See, Abrahamsen *et al.* (1991) *J. Clin. Apheresis*, 6: 48-53; Carter *et al.* (1988) *J. Clin. Apheresis*, 4:113-117; Aebersold *et al.* (1988) *J. Immunol. Meth.*, 112: 1-7; Muul *et al.* (1987) *J. Immunol. Methods* 101:171-181 and Carter *et al.* (1987) *Transfusion* 27: 362-365.  
20 After a period of about 2-4 weeks in culture, the cells should number between  $1 \times 10^8$  and  $1 \times 10^{12}$ . In this regard, the growth characteristics of cells vary from patient to patient and from cell type to cell type. About 72 hours prior to reinfusion of the transduced cells, an  
25 aliquot is taken for analysis of phenotype, and percentage of cells expressing the therapeutic agent.

#### VIII. Kits for transducing cells.

In another embodiment, this invention provides kits for practice of the methods described herein. The kits preferably include phage expressing a heterologous  
30 binding domain and containing a heterologous nucleic acid (e.g., an expression cassette) that is to be delivered inside a target cell or a nucleic acid encoding such a phage. The nucleic acid can include restriction sites to facilitate insertion of a heterologous nucleic acid into an

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expression cassette. The assay kits can additionally include any of the other components described herein for the practice of the assays of this invention. Such materials preferably include, but are not limited to, helper phage, one or more bacterial or mammalian cell lines, buffers, antibiotics, labels, and the like.

5 In addition the kits may optionally include instructional materials containing directions (*i.e.*, protocols) disclosing the transformation methods described herein. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to  
10 electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

### EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed  
15 invention.

#### Example 1: Creation of a non-immune human Fab phage antibody library containing $10^9$ - $10^{11}$ members

Manipulation of previous  $10^7$  member phage display libraries revealed two major limitations: 1) expression levels of Fabs was too low to produce adequate material for  
20 characterization, and 2) the library was relatively unstable. These limitations are a result of creating the library in a phage vector, and the use of the cre-lox recombination system. We therefore decided that the best approach for this project was to create a very large scFv library using a phagemid vector. The goal was to produce a library at least 100 times larger than our previous  $3.0 \times 10^7$  member scFv library. The approach taken was to clone the  $V_H$   
25 and  $V_L$  library on separate replicons, combine them into an scFv gene repertoire by splicing by overlap extension, and clone the scFv gene repertoire into the phage display vector pHEN1. Human peripheral blood lymphocyte and spleen RNA was primed with IgM heavy chain constant region and, kappa and lambda light chain constant region primers and first  
30 strand cDNA synthesized. 1st strand cDNA was used as a template for PCR amplification of  $V_H$   $V_{Hk}$  and  $V_L$  gene repertoires.



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The  $V_H$  gene repertoires were cloned into the vector pUC119Sfi-Not as NcoI-NotI fragments, to create a library of  $8.0 \times 10^8$  members. The library was diverse by PCR fingerprinting. Single chain linker DNA was spliced onto the  $V_L$  gene repertoires using PCR and the repertoire cloned as an XhoI-NotI fragment into the vector pHENIXscFv to create a library of  $7.2 \times 10^6$  members. The  $V_H$  and  $V_L$  gene repertoires were amplified from their respective vectors and spliced together using PCR to create an scFv gene repertoire. The scFv gene repertoire was cloned as an NcoI-NotI fragment into the vector to create an scFv phage antibody library of  $7.0 \times 10^9$  members. The library was diverse as determined by BstNI fingerprinting.

To verify the quality of the library, phage were prepared and selected on 14 different protein antigens. The results are shown in Table 2. scFv antibodies were obtained against all antigens used for selection, with between 3 and 15 unique scFv isolated per

Table 2. Results of phage antibody library selections. For each antigen (column 1), the number and the percentage of positive clones selected (column 2) and the number of different antibodies isolated (column 3) is indicated

Protein antigen used for selection	Percentage (number) of ELISA positive clones	Number of different antibodies isolated
FGF Receptor ECD	69 (18/26)	15
BMP Receptor Type I ECD	50 (12/24)	12
Activin Receptor Type I ECD	66 (16/24)	7
Activin Receptor Type II ECD	66 (16/24)	4
Erb-B2 ECD	91 (31/34)	14
VEGF	50 (48/96)	6
BoNT/A	28 (26/92)	14
BoNT-A C-fragment	95 (87/92)	10
BoNT/B	10 (9/92)	5
BoNT/C	12 (11/92)	5
BoNT/E	9 (8/92)	3
Bungarotoxin	67 (64/96)	15
Cytochrome b5	55 (53/96)	5
Chlamydia trachomatis EB	66 (63/96)	7

antigen (average 8.7) (Table 2). This compares favorably to results obtained from smaller scFv libraries (1 to a few binders obtained against only 70% of antigens used for selection).

Affinities of 4 anti-ErbB-2 scFv and 4 anti-Botulinum scFv were measured using surface plasmon resonance in a BIAcore and found to range from  $4.0 \times 10^{-9}$  M to  $2.2 \times 10^{-10}$  M for the anti-ErbB2 scFv and  $2.6 \times 10^{-8}$  M to  $7.15 \times 10^{-8}$  M for the anti-Botulinum scFv (Table 3).

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scFv were highly specific for the antigen used for selection (Figure 2). The library could also be successfully selected on complex mixtures of antigen.

Table 3. Affinities and binding kinetics of anti-BoNT A C-fragment and anti-Erb-B2 scFv. Association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rate constants for purified scFvs were measured using surface plasmon resonance (BIAcore) and  $K_d$  calculated as ( $k_{off}/k_{on}$ ).

Specificity and clone	$K_d$ ( $\times 10^{-9}M$ )	$k_{on}$ ( $\times 10^5 M^{-1}s^{-1}$ )	$k_{off}$ ( $\times 10^{-3}s^{-1}$ )
ErbB-2 B7A	0.22	4.42	0.1
ErbB-2 G11D	0.48	2.19	0.11
ErbB-2 A11A	0.49	3.69	0.18
ErbB-2 F5A	4.03	1.62	0.65
BoNT-A 2A9	26.1	0.25	0.66
BoNT-A 2H6	38.6	2.2	8.5
BoNT-A 3F6	66.0	4.7	30.9
BoNT-A 2B6	71.5	1.1	7.8

For example, selection on *Chlamydia trachomatis* elementary bodies (the causative organism of Chlamydial disease) yielded seven that specifically recognized chlamydia (Table 2). The

scFv could be successfully used in a number of immunologic assays including ELISA, immunofluorescence, Western blotting, epitope mapping and immunoprecipitation. The number of binding antibodies for each antigen, and the affinities of the binding scFv are comparable to results obtained from the best phage antibody libraries (Table 4). Thus the library was established as a source of panels of human antibodies against any antigen with

Table 4. Comparison of protein binding antibodies selected from non-immune phage-display antibody libraries. \* For library type, N = V-gene repertoires obtained from V-genes rearranged in vivo; SS = semi-synthetic V-genes constructed from cloned V-gene segments and synthetic oligonucleotides encoding  $V_H$  CDR3. ND = not determined.

Library	Library size and type*	Number of protein antigens studied	Average number of antibodies per protein antigen	Number of affinities measured	Range of affinities for protein antigens $K_d$ ( $\times 10^{-9}M$ )
Marks <i>et al</i> (1991) <i>J. Mol. Biol.</i> 222: 581-597	$3.0 \times 10^7$ (scFv, N)	2	2.5	1	100-2000
Nissim <i>et al</i> (1994) <i>EMBO J.</i> 13: 692-698	$1.0 \times 10^8$ (scFv, SS)	15	2.6	ND	ND

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DeKruif <i>et al</i> (1995) <i>J. Mol. Biol.</i> 248: 97-105	$3.6 \times 10^8$ (scFv, SS)	12	1.9	3	100 - 2500-
Griffiths <i>et al</i> (1994) <i>EMBO J.</i> 13: 3245-3260	$6.5 \times 10^{10}$ (Fab, SS)	30	4.8	3	7 - 58
Vaughan <i>et al</i> (1996) <i>Nature Biotechnology.</i> 14: 309-314	$1.4 \times 10^{10}$ (scFv, N)	3	7.0	3	4.2 - 8.0
Present Examples	$6.7 \times 10^9$ (scFv, N)	14	8.7	8	0.22 - 71.5

These experiments demonstrate the creation of a high complexity human scFv phage antibody library from which a panel of high affinity human scFv can be generated against any purified antigen. Such a library is ideal for probing the surface of cells to  
 5 identify novel cell surface markers.

**Example 2: Uptake of scFV into cells by receptor mediated endocytosis and subsequent recovery.**

The  $7.0 \times 10^9$  member scFv phage antibody library described above was selected on the malignant breast tumor cell lines MB231 and ZR-75-1, both with and without  
 10 negative selections on the normal breast cell line HBL100. Similar results were obtained as described in section above. scFv were isolated that could not distinguish malignant from non-malignant cell lines.

To increase the specificity of selections, it was hypothesized that phage binding cell surface receptors could be taken up into cells by receptor mediated endocytosis  
 15 and could then be recovered from cells by lysing the cells. This assumed: 1) that phage could be internalized by receptor mediated endocytosis and 2) that phage could be recovered in the infectious state from within cells prior to lysosomal degradation. The ability to select for internalized phage antibodies would have two major benefits: 1) the identification of antibodies that bind to receptors capable of internalization and 2) an added level of  
 20 specificity in the selection process. Identification of antibodies which are internalized would be highly useful for many targeted therapeutic approaches where internalization is essential (e.g. immunotoxins, targeted liposomes, targeted gene therapy vectors and others).

**A) Receptor mediated internalization of F5 or C1 phage**

To determine proof of principle, we utilized C6.5 phage and C6.5 diabody  
 25 phage (see, copending application USSN 08/665,202). We have previously shown that C6.5

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scFv is internalized, but at a slow rate, and that the C6.5 diabody is somewhat better internalized (probably because it causes receptor dimerization). C6.5 phage, C6.5 diabody phage or an irrelevant anti-Botulinum phage were incubated with SKBR3 cells (ErbB2 expressing breast tumor cell line) at either 37° C or 4° C and non-internalized phage removed by sequential washing with PBS and low pH glycine buffer. The cells were then permeabilized and biotinylated anti-M13-antibody added followed by streptavidin Texas Red. Cells were then examined by using a confocal microscope. Both C6.5 phage and C6.5 diabody phage were observed within the cytoplasm). Approximately 1% of cells had internalized C6.5 phage and 20% of the cells had internalized C6.5 diabody phage. There was no internalization of the anti-Botulinum phage.

To determine if infectious phage could be specifically taken up and recovered from within cells, C6.5 phage or C6.5 diabody phage were incubated with SKBR3 cells at 37° C. Non bound phage were removed by washing with PBS and phage bound to the cell surface were eluted by washing twice with low pH glycine. The cells were then lysed and each fraction (the first and second glycine washes and the cytoplasmic fraction) used to infect *E. coli* TG1. Twenty times (C6.5) or 30 times (C6.5 diabody) more phage were bound to the cell surface than the anti-Botulinum phage (glycine 1 wash) (Table 5). After the second glycine wash, the titre of infectious phage from the cell surface decreased, indicating that washing was effective at removing surface bound phage (Table 5). After cell lysis, the titer increased more than 10 fold (C6.5 phage) or 50 fold (C6.5 diabody phage) from the second glycine wash. We believe this titre represents phage recovered from inside the cell. Recovery of phage from inside the cell was 100 times higher for ErbB2 binding C6.5 than for anti-Botulinum phage and 200 fold higher for C6.5 diabody phage (Table 5).

Table 5. Titer of cell surface bound phage and internalized phage.  $5.0 \times 10^{11}$  phage (anti-Botulinum or anti-ErbB2) were incubated with approximately  $1.0 \times 10^5$  ErbB2 expressing SKBR3 cells at 37°C. Cells were washed 10 times with PBS and surface bound phage eluted with two low pH glycine washes. The cells were then washed once with PBS and the cells lysed to release internalized phage. The phage titer was then determined for each of the glycine washes and for the lysed cell fraction by infection of *E. coli* TG1.

Phage specificity	1st glycine wash	2nd glycine wash	Lysed cell fraction
anti-Botulinum	$6.0 \times 10^5$	$1.0 \times 10^5$	$6.0 \times 10^5$
Anti-ErbB2 (C6.5 scFv)	$1.2 \times 10^7$	$5.2 \times 10^6$	$6.8 \times 10^7$
Anti-ErbB2 (C6.5 diabody)	$1.8 \times 10^7$	$2.8 \times 10^6$	$1.7 \times 10^7$

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Taken together, the results indicate that: 1) phage binding cell surface receptors can be taken up by cells and the infectious phage recovered from the cytoplasm. The amount of uptake is significantly greater than uptake of non-binding phage, and the 100 to 200 fold difference is well within the range that would allow enrichment from a library. What is unknown from the results is whether the phage antibodies are mediating receptor mediated internalization or whether they are merely taken up after binding by membrane turnover.

#### B) Selection and characterization of internalizing antibodies from a phage antibody library

The results described above encouraged us to attempt selection of the phage antibody library described above to identify new phage antibodies that were internalized. Phage antibodies were rescued from the library and selected on SKBR3 cells. For selection, phage were incubated with cells at 37°C, non-binding phage removed by washing cells with PBS and phage bound to cell surface antigens removed by sequential washes with low pH glycine. Cells were then lysed to release internalized phage and the lysate used to infect *E. coli* TG1 to prepare phage for the next round of selection. Three rounds of selection were performed. One hundred clones from each round of selection were analyzed for binding to SKBR3 cells and to ErbB2 extracellular domain by ELISA. We hypothesized that we were likely to obtain binders to ErbB2 since SKBR3 cells are known to express high levels and ErbB2 is a receptor which is known to be internalized. After each round of selection, the titer of phage recovered from the cytoplasm increased (Table 6). After the third round, 45% of the clones were positive SKBR3 cell binding and 17% bound ErbB2 (Table 6).

Table 6. Results of selection of a phage antibody library for internalization. For each round of selection, the titer of phage in lysed cells, number of cells lysed and number of phage per cell is indicated. After the third round, individual clones were analyzed for binding to SKBR3 cells by ELISA and to ErbB2 ECD by ELISA.

Round of selection	# of phage in cell lysate	# of cells lysed	# of phage/cell	% SKBR3 binders	% ErbB2 binders
1	$3.5 \times 10^4$	$2.8 \times 10^6$	0.013	ND	ND
2	$1.2 \times 10^5$	$2.8 \times 10^6$	0.038	ND	ND
3	$7.5 \times 10^6$	$2.8 \times 10^6$	3.75	45%	17%

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To estimate the number of unique binders, the scFv gene from ELISA positive clones was PCR amplified and fingerprinted by digestion with BstN1. Two unique restriction patterns were identified. The scFv genes were sequenced and 2 unique ErbB2 binding scFv identified. Similar analysis of SKBR3 ELISA positive clones that did not bind ErbB2 identified an additional 11 unique scFv.

To verify that phage antibodies were specific for SKBR3 cells, phage were prepared from each unique clone and analyzed for binding to SKBR3 cells (high ErbB2 expression) as well as 2 other epithelial tumor cell lines (SK-OV-3, moderate ErbB2 expression and MCF7, low ErbB2 expression) and a normal breast cell line (HS578B). Each unique clone specifically stained tumor cell lines but not the normal breast cell line.

SKBR3 and MCF7 cells were incubated with phage antibodies C6.5 (positive control), 3TF5 and 3GH7. The latter two clones were isolated from the library, with 3TF5 binding ErbB2 and the antigen bound by 3GH7 unknown. All 3 phage antibodies intensely stain SKBR3 cells (the selecting cell line and high ErbB2 expresser. C6.5 phage weakly stain MCF7 cells (low ErbB2 expressor). The anti-ErbB2 clone 3TF5 from the library stains MCF7 cells much more intensely than C6.5, as does 3GH7.

SKBR3, SK-OV-3, MCF7 and HST578 cells were studied using native purified scFv 3TF5 and 3GH7. For these studies, the scFv genes were subcloned into a vector which fuses a hexahistidine tag to the scFv C-terminus. scFv was then expressed, harvested from the bacterial periplasm and purified by immobilized metal affinity chromatography. The two scFv intensely stain SKBR3 cells, and do not stain the normal breast cell line HST578. There is minimal staining of the low ErbB2 expressing cell line MCF7 and intermediate staining of SK-OV-3 cells (moderate ErbB2 expresser). In general, the intensity of staining is less than seen with phage. This is to be expected since the secondary antibody for phage staining recognizes the major coat protein (2500 copies/phage) resulting in tremendous signal amplification.

The anti-ErbB2 phage antibody 3TF5 was studied further to determine if it was indeed internalized. This antibody was selected for initial study since its internalization could be compared to ErbB2 binding C6.5.  $5.0 \times 10^{11}$  3TF5 or C6.5 phage were incubated with SKBR3 cells at 37°C or at 4°C. After washing with PBS, 3TF5 phage stained cells more intensely than C6.5 phage. After washing with low pH glycine, confocal microscopy revealed that 3TF5 phage were internalized by greater than 95% of cells, while C6.5 was

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internalized by only a few percent of cells. Incubation of either antibody at 4°C led to no internalization.

The native purified 3TF5 scFv was similarly analyzed and was also efficiently internalized by SKBR3 cells. It should be noted that the native 3TF5 scFv existed only as a monomer with no appreciable dimerization or aggregation as determined by gel filtration.

These experiments demonstrate that phage antibodies can be internalized by cells and recovered from the cytoplasm. Phage that bind an internalizing cell surface receptor can be enriched more than 100 fold over non-binding phage. This level of enrichment is greater than that achieved by selecting on the cell surface. We have applied this approach to library selection and isolated phage antibodies that bind and are internalized by SKBR-3 cells. Several of these antibodies bind to ErbB2, but are more efficiently internalized than antibodies such as C6.5 that were generated by selecting on pure antigen. Many other antibodies have been isolated that bind specifically to SKBR-3 and other breast tumor cell lines and are efficiently internalized. These antibodies should prove useful for tumor targeting and for identifying potentially novel internalizing tumor cell receptors.

Example 3: Increasing the affinity of antibody fragments with the desired binding characteristics by creating mutant phage antibody libraries and selecting on the appropriate breast tumor cell line.

Phage display has the potential to produce antibodies with affinities that cannot be produced using conventional hybridoma technology. Ultra high affinity human antibody fragments could result in excellent tumor penetration, prolonged tumor retention, and rapid clearance from the circulation, leading to high specificity. We therefore undertook a series of experiments to develop methodologies to generate ultra high affinity human antibody fragments. Experiments were performed to answer the following questions: 1) What is the most effective way to select and screen for rare higher affinity phage antibodies amidst a background of lower affinity binders; 2) What is the most effective means to remove bound phage from antigen, to ensure selection of the highest affinity phage antibodies; 3) What is the most efficient techniques for making mutant phage antibody libraries (random mutagenesis or site directed mutagenesis; 4) What region of the antibody molecule should be selected for mutagenesis to most efficiently increase antibody fragment affinity.

To answer these questions, we studied the human scFv C6.5, which binds the extracellular domain (ECD) of the tumor antigen ErbB-2 (32) with a  $K_d$  of  $1.6 \times 10^{-8}$  M and  $k_{off}$  of  $6.3 \times 10^{-3} \text{ s}^{-1}$  (Schier *et al.* (1995) *Immunotechnology*, 1: 63-71). Isolation and characterization of C6.5 is described briefly below and in detail in copending application USSN 08/665,202).

Despite excellent tumor:normal tissue ratios *in vivo*, quantitative delivery of C6.5 was not adequate to cure tumors in animals using radioimmunotherapy (Schier *et al.* (1995) *Immunotechnology*, 1: 63-71). To improve the quantitative delivery of antibody to tumor, the affinity of C6.5 was increased. First, techniques were developed that allowed selection of phage antibodies on the basis of affinity, rather than differential growth in *E. coli* or host strain toxicity (Schier *et al.* (1996) *J. Mol. Biol.* 255: 28-43; Schier *et al.* (1996) *Gene* 169: 147-155; Schier *et al.* (1996) *Human antibodies and hybridomas* 7: 97-105). Next, we determined which locations in the scFv gene to mutate to achieve the greatest increments in affinity (Schier *et al.* (1996) *J. Mol. Biol.* 255: 28-43; Schier *et al.* (1996) *Gene*; Schier *et al.* (1996) *J. Mol. Biol.* 263: 551-567). Random mutagenesis did not yield as great an increment in affinity as site directed mutagenesis of the complementarity determining regions (CDRs) that contain the amino acids which contact antigen. Results from diversifying the CDRs indicated that: 1) the greatest increment in affinity was achieved by mutating the CDRs located in the center of the binding pocket ( $V_L$  and  $V_H$  CDR3); 2) half of the CDR residues have a structural role in the scFv and when mutated return as wild-type; and 3) these structural residues can be identified prior to library construction by modeling on a homologous atomic crystal structure. These observations led to development of a generic strategy for increasing antibody affinity where mutations are randomly introduced sequentially into  $V_H$  and  $V_L$  CDR3, with conservation of residues postulated to have a structural role by homology modeling (Schier *et al.* (1996) *J. Mol. Biol.* 263: 551-567). Using this approach, the affinity of C6.5 was increased 1200 fold to a  $K_d$  of  $1.3 \times 10^{-11}$  M (*Id.*).

Biodistribution studies revealed a close correlation between affinity and the percent injected dose of scFv/gram of tumor (%ID/g) at 24 hours (Adams *et al.* (1998) *Cancer Res.* 58: 485-490). The greatest degree of tumor retention was observed with  $^{125}\text{I}$ -C6ML3-9 (1.42 %ID/g,  $K_d = 1.0 \times 10^{-9}$  M). Significantly less tumor retention was achieved with  $^{125}\text{I}$ -C6.5 (0.80 %ID/g,  $K_d = 1.6 \times 10^{-8}$ ) and C6G98A (0.19 %ID/g,  $K_d = 3.2 \times 10^{-7}$  M).



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The tumor:normal organ ratios also reflected the differences in affinity, e.g. tumor:blood ratios of 17.2, 13.3, 3.5 and 2.6, and tumor to liver ratios of 26.2, 19.8, 4.0 and 3.1 for C6ML3-9, C6.5 and C6G98A respectively at 24 hours. Studies of the higher affinity scFv are pending. The results demonstrate our ability to increase antibody affinity to values not achievable from hybridoma technology and confirm the importance of affinity in tumor targeting

#### Example 4: Preclinical development of C6.5 based breast cancer therapies

Two approaches have been collaboratively pursued to develop C6.5 based breast cancer therapies. In one collaboration, C6.5 based molecules are being engineered for radioimmunotherapy. To increase quantitative tumor delivery and retention of antibody fragment, dimeric scFv 'diabodies' were created by shortening the linker between the V<sub>H</sub> and V<sub>L</sub> domains from 15 to 5 amino acids. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalently bound dimer with two binding sites. *In vitro*, diabodies produced from the V-genes of C6.5 have a significantly higher apparent affinity and longer retention on the surface of SK-OV-3 cells compared to C6.5 scFv (T<sub>1/2</sub> > 5 hr vs. 5 min) (Adams *et al.* (1998) *Brit. J. Cancer.*). Biodistribution studies of C6.5 diabody revealed 6.5 %ID/g tumor at 24 hours compared to only 1 %ID/g for C6.5 scFv. When diabody retentions were examined over 72 hours and cumulative area under the curve (AUC) values determined, the resulting tumor:organ AUC ratios were greater than reported for other monovalent or divalent scFv molecules. The therapeutic potential of these molecules is being examined in radioimmunotherapy studies in nude mice. Since *in vivo* characterization of c6.5 based molecules was not formally one of the technical objectives, we are continuing to use the affinity mutants of C6.5 and C6.5 based diabodies to study the relationship between antibody affinity, size and valency and specific tumor targeting as part of NIH R01 1 CA65559-01A1.

In another collaboration, C6.5 based molecules are being used to target doxorubicin containing stealth liposomes to ErbB2 expressing breast cancers (Kirpotin *et al.* (1997) *Biochemistry*. 36: 66-75). To facilitate chemical coupling of the scFv to liposomes, the C6.5 gene was subcloned into an *E. coli* expression vector resulting in addition of a free cysteine residue at the C-terminus of the scFv. Purified C6.5cys scFv was conjugated to liposomes and *in vitro* uptake determined using SKBR3 cells. Total uptake was 3.4 mmol

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phospholipid/10<sup>6</sup> cells at 6 hour, with 70% of the uptake internalized. The uptake is comparable to that achieved using the 4D5 anti-HER2 Fab' from Genentech. There was no uptake of unconjugated liposomes. The results indicate that C6.5 binds to a ErbB2 epitope that results in internalization at a rate comparable to the best internalizing antibody produced from hybridomas (4D5). *In vivo* therapy studies in scid mice indicated that C6.5 targeted liposomes caused a greater degree of tumor regression and a higher cure rate than untargeted liposomes or a combination of untargeted liposomes and systemic 4D5 antibody.

### Conclusions

The experiments described herein establish that A large ( $7.0 \times 10^9$  member) phage antibody library has been created which can provide panels of human antibodies to purified antigens with affinities comparable to the affinities of antibodies produced by murine immunization. The phage antibodies binding cell surface receptors can be internalized by cells and recovered in an infectious state from within the cell. Methodologies were developed which permit enrichment of internalizing phage antibodies over non-internalizing antibodies more than 100 fold. These methodologies were then applied to select new scFv antibodies that bind to internalizing receptors on SKBR-3 cells. Several of these antibodies bind to ErbB2, but are internalized more efficiently than C6.5 based scFv. Many more antibodies bind to unknown internalizing receptors. All of these scFv bind specifically to SKBR-3 cells or related tumor cell lines. The results indicate that this selection approach is a powerful approach to generate antibodies that can distinguish one cell type (malignant) from another (non-malignant). Moreover, we have demonstrated that it is not only possible to select for binding, but to select for function (internalization). In the near term, we will further characterize the antibodies isolated with respect to specificity, and in the case of ErbB2 binding scFv, affinity. In the longer term we will use these reagents to:

- 1) study the effect of affinity and valency on the rate of internalization; and 2) identify the antigens bound using immunoprecipitation. It is likely that the results will lead to the identification of novel internalizing tumor cell surface receptors which will be useful therapeutic targets. If this approach proves useful, we plan on applying it to primary tumor cells and DCIS. We also intend to evaluate 3TF5 (ErbB2 binding scFv which is internalized faster than C6.5) for liposome targeting. It is possible that it will be more effective than C6.5

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In addition, the experiments demonstrate that methodologies for increasing antibody affinity *in vitro* to values not previously achieved *in vivo*. We have applied these methodologies to generate novel ErbB2 binding scFv.

#### Example 5: Selection of internalizing antibodies from phage libraries

5           Antibodies that bind cell surface receptors in a manner whereby they are endocytosed are useful molecules for the delivery of drugs, toxins or DNA into the cytosol of mammalian cells for therapeutic applications. Traditionally, internalizing antibodies have been identified by screening hybridomas. In this example, we studied a human scFv (C6.5) that binds ErbB2 to determine the feasibility of directly selecting internalizing antibodies  
10   from phage libraries and to identify the most efficient display format. Using wild type C6.5 scFv displayed monovalently on a phagemid, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single copies on a phagemid or multiple copies on phage, we define the role of affinity, valency, and display format on phage endocytosis and  
15   identify the factors that lead to the greatest enrichment for internalization. Phage displaying bivalent diabodies or multiple copies of scFv were more efficiently endocytosed than phage displaying monomeric scFv and recovery of infectious phage was increased by preincubation of cells with chloroquine. Measurement of phage recovery from within the cytosol as a function of applied phage titer indicates that it is possible to select for endocytosable  
20   antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of  $10^9$ .

#### A) Introduction

Growth factor receptors are frequently overexpressed in human carcinomas and other diseases and thus have been utilized for the development of targeted therapeutics.  
25   The *HER2/neu* gene, for example, is amplified in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (Slamon *et al.* (1989) *Science* 244: 707-712) leading to the overexpression of the corresponding growth factor receptor ErbB2. Targeting of ErbB2 overexpressing cells has been accomplished primarily using anti-ErbB2 antibodies in different formats, including conjugation to liposomes containing chemotherapeutics  
30   (Kirpotin *et al.* (1997). *Biochem.* 36: 66-75), fusion to DNA carrier proteins delivering a toxic gene (Forminaya and Wels (1996) *J. Biol. Chem.* 271: 10560-10568), and direct fusion

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to a toxin (Altenschmidt *et al.* (1997) *Int. J. Cancer* 73: 117-124). For many of these targeted approaches, it is necessary to deliver the effector molecule across the cell membrane and into the cytosol. This can be accomplished by taking advantage of normal growth factor receptor biology; growth factor binding causes receptor activation via homo- or heterodimerization, either directly for bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor mediated endocytosis (Ullrich and Schlessinger (1990) *Cell* 61: 203-212). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cytosol. In general, this requires a bivalent antibody capable of mediating receptor dimerization (Heldin (1995) *Cell* 80: 213-223; Yarden (1990) *Proc. Natl. Acad. Sci. USA* 87: 2569-2573). In addition, the efficiency with which antibodies mediate internalization differs significantly depending on the epitope recognized (Yarden (1990) *Proc. Natl. Acad. Sci. USA* 87: 2569-2573; Hurwitz *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 3353-3357.). Thus for some applications, such as liposomal targeting, only antibodies which bind specific epitopes are rapidly internalized and yield a functional targeting vehicle.

Currently, antibodies which mediate internalization are identified by screening hybridomas. Alternatively, it might be possible to directly select internalizing antibodies from large non-immune phage libraries (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95: 6157-6162) by recovering infectious phage particles from within cells after receptor mediated endocytosis, as reported for peptide phage libraries (Hart *et al.* (1994) *J. Biol. Chem.* 269: 12468-12474; Barry *et al.* (1996) *Nat. Med.* 2: 299-305). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597; Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95: 6157-6162.). We hypothesized that such monovalent display was unlikely to lead to efficient receptor crosslinking and phage internalization. To determine the feasibility of selecting internalizing antibodies and to identify the most efficient display format, we studied a human scFv (C6.5) which binds ErbB2 (13). Using wild type C6.5 scFv, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single or multiple copies on the phage surface, we define the role of affinity, valency, and display

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format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. The results indicate that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of  $10^9$  members.

## 5      A) Material and methods

### 1) Cells

The SKBR3 breast tumor cell line was obtained from ATCC and grown in RPMI media supplemented with 10% FCS (Hyclone) in 5% CO<sub>2</sub> at 37°C.

### 2) Antibodies and antibody phage preparations

10            The C6.5 scFv phage vector was constructed by subcloning the C6.5 gene as a *Sfi* I/*Not* I fragment from scFv C6.5 pHEN1 (Schier *et al.* (1995) *Immunotechnology* 1: 63-71) into the phage vector *fd/Sfi* I/*Not* I (a gift of Andrew Griffiths, MRC Cambridge, UK). The C6.5 diabody phagemid vector was constructed by subcloning the C6.5 diabody gene (Adams *et al.* (1998) *Brit. J. Cancer*. 77: 1405-1412, 1998) as a *Nco*I/*Not*I fragment into  
15      pHEN1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137). The anti-botulinum scFv phagemid (clone 3D12) (Amersdorfer *et al.* (1997) *Infection and Immunity* . 65: 3743-3752) C6.5 scFv phagemid (Schier *et al.* (1995) *Immunotechnology* 1: 63-71) and scFv C6ML3-9 scFv phagemid (Schier *et al.* (1996) *J. Mol. Biol.* 263: 551-567) in pHEN1 have  
20      been previously described. Phage were prepared (Sambrook *et al.* (1990). *Molecular cloning- a laboratory manual.*, New York: Cold Spring Harbor Laboratory) from the appropriate vectors and titered on *E. coli* TG1 as previously described (Marks *et al.* (1991) *J. Mol. Biol.* 222: 581-597) using ampicillin (100 µg/ml) resistance for titration of constructs in pHEN1 and tetracycline (50 µg/ml) for titration of constructs in *fd*. Soluble C6.5 scFv, C6.5 diabody and anti-botulinum scFv were expressed from the vector pUC119mycHis (Schier *et al.* (1995) *Immunotechnology* 1: 63-71) and purified by immobilized metal affinity  
25      chromatography as described elsewhere (*Id.*)).

### 3) Detection of internalized native antibody fragments and phage antibodies

SKBR3 cells were grown on coverslips in 6-well culture plates (Falcon) to 50% of confluency. Culture medium was renewed 2 hours prior to the addition of  $5 \cdot 10^{11}$

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cfu/ml of phage preparation (the phage preparation representing a maximum of 1/10 of the culture medium volume) or 20 µg/ml of purified scFv or diabody in phosphate buffered saline, pH 7.4 (PBS). After 2 hours of incubation at 37°C, the wells were quickly washed 6 times with ice cold PBS and 3 times for 10 minutes each with 4 mL of stripping buffer (50 mM glycine pH 2.8, 0.5 M NaCl, 2M urea, 2% polyvinylpyrrolidone) at RT. After 2 additional PBS washes, the cells were fixed in 4% paraformaldehyde (10 minutes at RT), washed with PBS, permeabilized with acetone at -20°C (30 seconds) and washed again with PBS. The coverslips were saturated with PBS-1% BSA (20 min. at RT). Phage particles were detected with biotinylated anti-M13 immunoglobulins (5 Prime-3 Prime, Inc, diluted 300 times) (45 min. at RT) and Texas red-conjugated streptavidin (Amersham, diluted 300 times) (20 min. at RT). Soluble scFv and diabodies containing a C-terminal myc peptide tag were detected with the mouse mAb 9E10 (Santa Cruz Biotech, diluted 100 times) (45 min. at RT), anti-mouse biotinylated immunoglobulins (Amersham, diluted 100 times) and Texas red-conjugated streptavidin. Optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Alternatively, slides were analyzed with a Zeiss Axioskop UV fluorescent microscope.

#### 4) Recovery and titration of cell surface bound or internalized phage

Subconfluent SKBR3 cells were grown in 6-well plates. Culture medium was renewed 2 hours prior to the experiment. Cells were incubated for varying times with different concentrations of phage preparation at 37°C. Following PBS and stripping buffer washes, performed exactly as described above for detection of internalized native antibody fragments and phage antibodies, the cells were washed again twice with PBS and lysed with 1 mL of 100 mM triethylamine (TEA). The stripping buffer washes and the TEA lysate were neutralized with 1/2 volume of Tris-HCl 1M, pH 7.4. For some experiments, cells were trypsinized after the three stripping buffer washes, collected in a 15 ml Falcon tube, washed twice with PBS and then lysed with TEA. In experiments performed in the presence of chloroquine, SKBR3 cells were preincubated for two hours in the presence of complete medium containing 50 µM chloroquine prior to the addition of phage. Corresponding control samples in the absence of chloroquine were prepared at the same time. For all experiments, phage were titrated on *E. coli* TG1 as described above.

## B) Results

### 1) The model system utilized to study phage antibody internalization

The human anti-ErbB2 scFv C6.5 was obtained by selecting a human scFv phage antibody library on recombinant ErbB2 extracellular domain (13). C6.5 scFv binds  
5 ErbB2 with a  $K_d = 1.6 \times 10^{-8}$  M and is a stable monomeric scFv in solution with no tendency to spontaneously dimerize or aggregate (Schier *et al.* (1995) *Immunotechnology* 1: 63-71). To determine the impact of affinity on internalization, we studied a scFv (C6ML3-9) which differs from C6.5 by 3 amino acids (Schier *et al.* (1996) *J. Mol. Biol.* 263: 551-567). C6ML3-9 scFv is also a stable monomer in solution and binds the same epitope as C6.5 scFv  
10 but with a 16 fold lower  $K_d$  ( $1.0 \times 10^{-9}$  M) (Schier *et al.* (1996) *J. Mol. Biol.* 263: 551-567; Adams *et al.* (1998) *Cancer Res.* 58: 485-490). Since receptor homodimerization appears to typically be requisite for antibody internalization we also studied the dimeric C6.5 diabody (Adams *et al.* (1998) *Brit. J. Cancer.* 77: 1405-1412, 1998). Diabodies are scFv dimers where each chain consists of heavy ( $V_H$ ) and light ( $V_L$ ) chain variable domains connected  
15 using a peptide linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (Holliger *et al.* (1993) *Proc. Natl. Acad. Sci.* 90: 6444-6448). The C6.5 diabody was constructed by shortening the peptide linker between the Ig  $V_H$  and  $V_L$  domains from 15 to 5 amino acids and binds ErbB2  
20 on SKBR3 cells bivalently with a  $K_d$  approximately 40 fold lower than C6.5 ( $4.0 \times 10^{-10}$  M) (Adams *et al.* (1998) *Brit. J. Cancer.* 77: 1405-1412, 1998).

Native C6.5 scFv and C6.5 diabody was expressed and purified from *E. coli* and analyzed for endocytosis into ErbB2 expressing SKBR3 breast tumor cells by immunofluorescent confocal microscopy. As expected, monomeric C6.5 scFv is not  
25 significantly internalized whereas the dimeric C6.5 diabody can be detected in the cytoplasm of all cells visualized.

For subsequent experiments, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19: 4133-4137). This should yield phagemid predominantly  
30 expressing a single scFv or diabody-pIII fusion after rescue with helper phage (Marks *et al.* (1992) *J. Biol. Chem.* 267: 16007-16010) (Figures 2A and 2B). Diabody phagemid display a

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bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule (Figure 2B). The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (Figure 2C). The human breast cancer cell line SKBR3 was used as a target cell line for endocytosis. Its surface ErbB2 density is approximately  $1.0 \times 10^6$  per cell (Hynes *et al.* (1989) *J. Cell. Biochem* 39: 167-173).

### 2) C6.5 phagemids are endocytosed by human cells

C6.5 scFv phagemids were incubated for 2 hours with SKBR3 cells grown on coverslips at 37°C to allow active internalization. Cells were extensively washed with PBS to remove non specific binding and washed an additional three times with high salt and low pH (stripping) buffer to remove phage specifically bound to cell surface receptors. Internalized phagemid were detected with a biotinylated M13 antiserum recognizing the major coat phage protein pVIII. An anti-botulinum toxin phagemid was used as a negative control. Staining was analyzed by using immunofluorescent microscopy. Approximately 1% of the cells incubated with C6.5 scFv phagemid showed a strong intracellular staining consistent with endosomal localization while no staining was observed for anti-botulinum phagemid. Furthermore, no staining was seen if the incubation was performed for 2 hours at 4°C instead of 37°C (data not shown). Staining performed after the PBS washes but before washing with stripping buffer showed membrane staining of all the cells, indicating that multiple washes with stripping buffer is necessary to remove surface bound phagemids. The results also indicate that only a fraction of the cell bound phage are endocytosed.

### 3) Increased affinity and bivalency lead to increased phage endocytosis

We compared the internalization of C6.5 scFv, C6ML3-9 scFv and C6.5 diabody phagemid and C6.5 scFv phage using immunofluorescence. Both C6ML3-9 scFv and C6.5 diabody phagemid as well as C6.5 scFv phage yielded increased intensity of immunofluorescence observed at the cell surface compared to C6.5 scFv phagemid. For C6ML3-9 scFv phagemid, approximately 10% of the cells showed intracellular fluorescence after 2 hours of incubation. This value increased to approximately 30% of cells for the dimeric C6.5 diabody phagemid and 100% of cells for multivalent C6.5 scFv phage.



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**3) Infectious phage can be recovered from within the cell and their titre correlates with the level of uptake observed using immunofluorescence**

To determine if infectious phage antibody particles could be recovered from within the cell, we incubated approximately  $5.0 \times 10^5$  SKBR-3 cells for 2 hours at 37°C with  $3.0 \times 10^{11}$  cfu of the different phagemid or phage. Six PBS washes were used to remove non-specifically bound phage and specifically bound phage were removed from the cell surface by three consecutive washes with stripping buffer (washes I, II and III respectively, Table 7). The cells were then lysed with 1 mL of a 100 mM triethylamine solution (TEA) (representing the intracellular phage). The three stripping washes and the cell lysate were neutralized and their phage titer was determined by infection of *E. coli* TG1. The titers of phage recovery are reported in Table 7.

**Table 7: Titration of membrane bound and intracellular phage.**  $3.0 \times 10^{11}$  cfu of monovalent C6.5 scFv phagemid, 16 fold higher affinity monovalent C6ML3-9 scFv phagemid, bivalent C6.5 diabody phagemid or multivalent C6.5 fd phage were incubated with sub confluent SKBR3 cells for 2 hours at 37°C. Cells were washed 6 times with PBS, 3 times with stripping buffer and then lysed to recover intracellular phage. The various fractions were neutralized and the phage titered. The total number of cfu of each fraction is reported. Non specific anti-botulinum phagemid were used to determine non specific recovery.

Phage Antibody	Cell Surface Phage Titer ( $\times 10^{-5}$ )			Intracellular Phage Titer ( $\times 10^{-5}$ )
	1st Wash	2nd Wash	3rd Wash	
Anti-botulinum phagemid	280	36	2.8	15
C6.5 scFv phagemid	600	96	7.6	52
C6ML3-9 scFv phagemid	2500	140	32	270
C6.5 diabody phagemid	1800	120	13	450
C6.5 scFv phage	2300	620	56	2200

Considerable background binding was observed in the first stripping wash for the anti-botulinum phage even after 6 PBS washes ( $2.8 \times 10^7$  cfu, Table 7). This value likely represents phage non-specifically bound to the cell surface as well as phage trapped in the extracellular matrix. The amount of surface bound phage increased only 2.1 fold above this background for C6.5 scFv phagemid (Tables 7 and 9). With increasing affinity and avidity of the displayed C6.5 antibody fragment, the titer of cell surface bound phagemid or phage

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increased (Table 7). The titer of phage in the consecutive stripping washes decreased approximately 10 fold with each wash. These additional stripping washes led to a minor increase in the titer of specific phage eluted compared to the background binding of the anti-botulinum phage (2.7 fold for C6.5 scFv phagemid to 20 fold for C6.5 scFv phage, Table 9). The only exception was the titer of the C6.5 diabody phagemid, where the ratio actually decreased from 6.4 fold to 4.6 fold. This is likely due to the fact that in the diabody the V<sub>H</sub> and V<sub>L</sub> domains that comprise a single binding site are not covalently attached to each other via the peptide linker. This increases the likelihood that a stringent eluent (like glycine) could dissociate V<sub>H</sub> from V<sub>L</sub> and abrogate binding to antigen.

**Table 9: Specific enrichment of anti-ErbB2 phage compared to anti-botulinum phage.**  
 \*The titers of anti-ErbB2 phage are divided by the titers of the anti-botulinum phage (Table 7) to derive an enrichment ratio for specific vs nonspecific binding or internalization. \*\*The titer of intracellular phage is divided by the titer of cell surface bound phage (Table 7) to derive the ratio of internalized phage vs surface bound phage.

Phage Antibody	Anti-ErbB2 /Anti-Botulinum Phage Titer Ratio*			Intracellular/ Cell Surface Phage Ratio**
	Cell surface (1st Wash)	Cell surface (3rd Wash)	Intracellular	
C6.5 scFv phagemid	2.14	2.7	3.5	6.8
C6ML3-9 scFv phagemid	8.9	11.4	18	8.4
C6.5 diabody phagemid	6.4	4.6	30	35
C6.5 scFv phage	8.2	20	146	39

Three stripping washes were required to ensure that the titer of phage recovered after cell lysis was greater than the titer in the last stripping wash (Table 7). We presumed that after three stripping washes, the majority of the phage eluted represented infectious particles from within the cell rather than from the cell surface. In fact, since the cell lysate titer observed with non-specific anti-botulinum phage was considerable (1.5 x 10<sup>6</sup>) and greater than observed in the last stripping wash, it is likely that many phage remain trapped within the extracellular matrix and relatively inaccessible to the stripping buffer washes. Some anti-botulinum phage might also be non-specifically endocytosed by cells, but this is likely to be a small amount given the immunofluorescence results. The titer of phage in the TEA fraction increased with increasing affinity and avidity of C6.5, with the

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highest titers observed for the dimeric C6.5 diabody phagemid and the multivalent C6.5 scFv phage (Table 7). The values represent a 30 fold (C6.5 diabody phagemid) and 146 fold (C6.5 scFv phage) increase in titer compared to the anti-botulinum phage (Table 7). We have presumed that the increase in the phage titer in the cell lysate compared to the last stripping wash is due to endocytosed phage. In fact, some of these phage could have come from the cell surface or intracellular matrix. While this could be true for a fraction of the phage from the cell lysate, the immunofluorescence results indicate that at least some of the phage are endocytosed. One indicator of the relative fraction of endocytosed phage for the different C6.5 molecules is to compare the amount of phage remaining on the cell surface prior to cell lysis (last stripping wash) with the amount recovered after cell lysis. This ratio shows only a minor increase for monovalent C6.5 scFv or C6ML3-9 scFv phagemid (6.8 and 8.4 fold respectively) compared to anti-botulinum phagemid (5.4) (Table 9). In contrast the ratios for dimeric C6.5 diabody phagemid and multivalent C6.5 scFv phage increase to a greater extent (35 and 39 respectively) compared to anti-botulinum phagemid.

#### 4) Increasing the enrichment ratios of specifically endocytosed phage

The results above indicate that phage antibodies can undergo receptor mediated endocytosis and remain infectious in a cell lysate. Selection of internalized phages from a phage library requires the optimization of the method to increase enrichment of specifically internalized phages over non-internalized phage. Two parameters can be improved: (1) reduction of the recovery of non-specific or non-internalized phage and (2) preservation of the infectivity of internalized phage. To examine these parameters, we studied wild-type C6.5 scFv phagemid. We chose this molecule because it was clearly endocytosed based on confocal microscopy, yet was the molecule undergoing the least degree of specific endocytosis. C6.5 scFv phagemid also represents the most commonly utilized format for display of non-immune phage antibody libraries (single copy pIII in a phagemid vector) and has an affinity (16 nM) more typical of Kd's of scFv from such libraries than the affinity matured C6ML3-9 scFv (Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95: 6157-6162; Vaughan *et al.* (1996) *Nature Biotech.* 14: 309-314).

##### a) Reducing the background of non-internalized phage

To reduce the background of non-specific phage recovery, we studied the effect of trypsinizing the cells prior to TEA lysis. This should remove phage trapped in the

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extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. For these experiments, C6.5 scFv phagemid ( $5.0 \times 10^8$  ampicillin resistant cfu) were mixed with a 1000 fold excess of wild type fd phage ( $5.0 \times 10^{11}$  tetracycline resistant cfu). After incubation of phagemid with SKBR-3 cells for 2 hours at 37°C, cells were washed with PBS and three times with stripping buffer. Cells were then directly lysed with TEA or treated with trypsin, washed twice with PBS and then lysed with TEA. Phagemid in the first stripping wash and the cell lysate were titered by infection of *E. coli* TG1 and plated on ampicillin and tetracycline plates. The titer of fd phage and C6.5 scFv phagemid recovered from the cell surface was comparable for the two experimental groups (Figure 3). The ratio of fd phage/C6.5 scFv phagemid in the cell surface fractions (160/1 and 250/1) yields a 4 to 6 fold enrichment achieved by specific cell surface binding from the initial 1000 fold ratio. Without trypsinization, the ratio of fd phage /C6.5 scFv phagemid in the cell lysate increases only 6.1 fold; in contrast, the ratio increases 209 fold with trypsinization (Figure 3). This results from a 60 fold reduction in non-specific binding with only a minor reduction in the amount of specific phage recovery (Figure 3).

#### b) Improving the recovery of infectious internalized phage

To increase the recovery of infectious internalized phage, we studied whether prevention of lysosomal acidification through the use of chloroquine would protect endocytosed phages from endosomal degradation (Barry *et al.* (1996) *Nat. Med.* 2: 299-305). SKBR3 cells were incubated with chloroquine and either C6.5 scFv phagemid or anti-botulinum phagemid. Cell lysates were titered at various time points to determine the number of intracellular phagemid. C6.5 scFv phagemid were present at the 20 minute time point and the amount of phagemid was comparable with or without the addition of chloroquine. At later time points, approximately twice as much infectious phagemid was recovered with the use of chloroquine. In contrast, much lower amounts of anti-botulinum phage were present and chloroquine had no effect on the titer, suggesting that the phagemid result from non-specific surface binding rather than non-specific endocytosis into endosomes. Overall, the results indicate that prevention of lysosomal acidification increases the amount of infectious phage recovered for incubations longer than 20 minutes.

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### 5) Recovery of internalized phage at low phage concentrations

Only very large phage antibody libraries containing more than  $5.0 \times 10^9$  members are capable of generating panels of high affinity antibodies to all antigens (10, 23, 24). Since phage can only be concentrated to approximately  $10^{13}$  cfu/ml, a typical phage preparation from a large library will only contain  $10^4$  copies of each member. Thus selection of libraries for endocytosis could only work if phage can be recovered when applied to cells at titers as low as  $10^4$ . We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the phage titer applied. SKBR3 cells were incubated with C6.5 scFv, C6ML3-9 scFv or C6.5 diabody phagemids or C6.5 scFv phage for 2 hours at 37°C. Cells were washed three times with stripping buffer, trypsinized and washed twice with PBS. Cells were lysed and intracellular phage titered on *E. coli* TG1. Phage recovery increased with increasing phage titer for all phage studied (Figure 5). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than  $3.0 \times 10^5$  (C6.5 scFv) to  $3.0 \times 10^6$  (C6ML3-9 scFv). This threshold decreased for bivalent and multivalent display ( $3.0 \times 10^4$  for C6.5 diabody phagemid and C6.5 scFv phage).

### C) Discussion

We demonstrate for the first time that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. Phage displaying anti-ErbB2 antibody fragments are specifically endocytosed by ErbB2 expressing SKBR3 cells, can be visualized within the cytosol and can be recovered in an infectious form from within the cell. When monovalent scFv antibody fragments were displayed monovalently in a phagemid system, recovery of internalized phage was only 3.5 to 18 fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30 to 146 fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (Yarden (1990) *Proc. Natl. Acad. Sci. USA*

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87: 2569-2573; Hurwitz *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 3353-3357). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv (Marks *et al.* (1992) *J. Biol. Chem.* 267: 16007-16010). The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by mammalian cells (Hart *et al.* (1994) *J. Biol. Chem.* 269: 12468-12474). Recovery of phage after endocytosis also increases the specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs non-specific surface binding range from 2 to 20 fold. These values are comparable to the approximately 10 fold enrichment reported by others for a single round of cell surface selection (Pereira *et al.* (1997) *J. Immunol. Meth.* 203: 11-24; Watters *et al.* (1997) *Immunotechnology* 3: 21-29). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5 to 146 fold.

Based on these results, selection of internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector. While no such libraries have been published, there are no technical barriers preventing their construction. Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor down regulation and growth inhibition (Hurwitz *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92: 3353-3357; Hudziak *et al.* (1989) *Mol. Cell. Biol.* 9: 1165-1172; Stancovski *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88: 8691-8698; Lewis *et al.* (1993) *Cancer Immunol.*

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*Immunother.* 37: 255-263), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

#### Example 6: Transfection of Cells.

The F5 scFv gene was removed from pHEN1-F5 by digestion of phagemid DNA with the restriction enzymes SfiI and NotI. A phage vector based on FdDOG1 (See prior Ref.), but modified to insert an SfiI site into the gene III leader sequence, was digested with SfiI and NotI and the digested F5 gene ligated into digested phage Fd vector DNA. Recombinant transformant were identified. *E. coli* containing the F5 recombinant phage were grown in culture to produce F5-Fd phage (see Maniatis for phage preparation). F5 phages were then used to infect *E. coli* harboring a phagemid which contains a mammalian promoter (CMV) followed by either the gene for  $\beta$ -galactosidase (pcDNA3.1/HisB/LacZ, In Vitrogen) or the gene for the enhanced green fluorescent protein (pN2EGFP, Clontech plasmid) and a eucaryotic polyadenylation sequence. Bacteria were grown overnight in the presence of tetracycline 15 ug/mL and either ampicillin 100 ug/mL (pcDNA3.1/HisB/LacZ containing bacteria) or Kanamycine 30 ug/mL (pN2EGFP containing bacteria). The phage prepared from the supernatant a mixture of F5-Fd coat contains either the reporter gene (about 50% of the phages) in a single strand format or the F5-Fd phage genome (about 50% of the phages). Incubation of ErbB2 positive cells 5.105 SKBR3 with  $10^7$  pfu the phage mix (Filtered twice through a 0.45 nm filter to sterility) allowed expression of the reporter gene in 1% of the cells. Cells incubated with an 10 time fold more negative control phage, *i.e.* reporter gene packaging in wild type Fd, showed no expression of the reporter genes. In an experiment where a mixed population of ErbB2 high (SKBR3) and ErbB2 low cells (MCF7) (Lewis *et al.* (1993) *Cancer Immunol Immunother* 37: 255-263) were incubated with the F5-Fd-EGFP phages for two days, we obtained the expression of the reporter gene only in erbB2 positive cells, cells being differentiated by their ErbB2 level by FACS.

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#### Example 7: Targeted gene delivery to mammalian cells by filamentous bacteriophage

In this example we show that prokaryotic viruses can be re-engineered to infect eukaryotic cells resulting in expression of a portion of the bacteriophage genome. Phage capable of binding mammalian cells expressing the growth factor receptor ErbB2 and undergoing receptor mediated endocytosis were isolated by selection of a phage antibody library on breast tumor cells and recovery of infectious phage from within the cell. As

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determined by Immunofluorescence, F5 phage were efficiently endocytosed into 100% of ErbB2 expressing SKBR3 cells. To achieve expression of a portion of the phage genome, F5 phage were engineered to package the green fluorescent protein (GFP) reporter gene driven by the CMV promoter. These phage when applied to cells underwent ErbB2 mediated endocytosis leading to GFP expression. GFP expression occurred only in cells overexpressing ErbB2, was dose dependent reaching 4% of cells after 60 hours and was detected with phage titers as low as  $2.0 \times 10^7$  cfu/ml (500 phage/cell). The results demonstrate that bacterial viruses displaying the appropriate antibody can bind to mammalian receptors and utilize the endocytic pathway to infect eukarotic cells resulting in viral gene expression. This represents a novel method to discover targeting molecules capable of delivering a gene intracellularly into the correct trafficking pathway for gene expression by directly screening phage antibodies. This should significantly facilitate the identification of appropriate targets and targeting molecules for gene therapy or other applications where delivery into the cytosol is required. This approach can also be adapted to directly select, rather than screen, phage antibodies for targeted gene expression. The results also demonstrate the potential of phage antibodies as an *in vitro* or *in vivo* targeted gene delivery vehicle.

## B) Materials and Methods

### 1) Anti-ErbB2 F5 scFv

An anti-ErbB2 scFv (F5) in the vector pHEN-1 (Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19(15): 4133-4137) (pHEN-F5) was obtained by selecting a non-immune phage antibody library (Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(11): 6157-6162) on ErbB2 expressing SKBR3 cells followed by screening for binding on recombinant ErbB2 extracellular domain (ECD). The native F5 scFv binds ErbB2 ECD with a  $K_d = 1.6 \times 10^{-7}$  M as determined by surface plasmon resonance in a BIAcore as previously described (Schier *et al.* (1996) *J. Mol. Biol.* 255(1): 28-43).

### 2) Phage and phagemid vectors

pcDNA3-GFP (6.1 Kbp) was obtained by subcloning the *Hind* III/*Not* I fragment of pN2EGFP (4.7 Kbp) (Clontech) into the pcDNA3-HisB/LacZ (Invitrogen) *Hind* III/*Not* I backbone. A fd-F5-phage vector was constructed by subcloning the *Sfi* I/*Not* I



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scFv-F5 insert from pHEN-1 into the *Sfi* I/*Not* I sites of fd-*Sfi*/*Not* (constructed from fd-tet-DOG (Clackson *et al.* (1991) *Nature* 352(6336): 624-628) by changing the *Apa*L1 cloning site in the gene III leader to *Sfi*L. The pHEN-F5-GFP phagemid vector (6.8 Kbp) was obtained by subcloning the 1.6 Kbp pN2EGFP blunted *Ase* I/*Afl* II fragment into the blunted *Eco*R I site of pHEN-F5. The orientation of the insert was analyzed by *Not* I restriction digest.

### 3) Cell line culture and transfection

SKBR3 and MCF7 were grown in RPMI complemented with 10% fetal bovine serum (FBS) (Hyclone). 50 % confluent SKBR3 cells grown in 6-well plates were transfected with 1 µg of DNA per well using Lipofectamine (GIBCO BRL) as recommended by the manufacturer. pN2EGFP dsDNA was prepared by alkaline lysis using the Maxiprep Qiagen Kit (Qiagen Inc.). ssDNA was extracted from 500 µl of phagemid preparation (see below) by 2 phenol extractions followed by ethanol precipitation. DNA was quantified by spectrophotometry with 1.0 A<sub>260</sub> nm equal to 40 µg/ml for ssDNA or 50 µg/ml for dsDNA. For GFP detection, cells were detached using a trypsin-EDTA mix (GIBCO BRL) and analyzed on a FACScan (Becton Dickinson).

### 4) Phagemid and phage preparation

pHEN-F5, pHEN-F5-GFP, pcDNA3-GFP or pN2EGFP phagemids were prepared from *E. coli* TG1 by superinfection with VCS-M13 helper phage (Stratagene) as previously described (Marks *et al.* (1991) *J. Mol. Biol.* 222(3): 581-597). Fd-F5-phage were prepared from *E. coli* TG1 as previously described (McCafferty *et al.* (1990) *Nature* 348(6301): 552-554). F5-GFP-phage and F5-LacZ-phage were prepared by superinfection of *E. coli* TG1 containing pcDNA3-GFP with fd-F5-phage. Virus particles were purified from the culture supernatant by 2 polyethylene glycol precipitations (Sambrook *et al.* (1990). *Molecular cloning- a laboratory manual*, Cold Spring Harbor Laboratory, New York) resuspended in phosphate buffered saline, pH 7.4 (PBS), filtered through a 0.45 µm filter and stored at 4°C. Alternatively, the preparations were submitted to an additional CsCl ultracentrifugation step (Smith and Scott (1993) *Meth. Enzymol.* 217: 228-257). The ratio of packaged helper phage DNA versus phagemid DNA was determined by titering (Sambrook *et al.*, *supra*.) the phage for ampicillin and kanamycin resistance (for helper

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phage rescued pHEN-F5) or ampicillin and tetracycline resistance (for fd-F5 phage rescued - pcDNA3-GFP).

### 5) Phage FACS

Cells were trypsinized, washed with PBS containing 1% FBS (FACS buffer) and resuspended at  $10^6$  cells/ml in the same buffer. The staining procedure was performed on ice with reagents diluted in FACS buffer. One hundred  $\mu$ l aliquots of cells were distributed in conical-96-well plate (Nunc), centrifuged at 300g and the cell pellets resuspended in 100  $\mu$ l of serial dilutions of phage or phagemid preparation and incubated for 1 hr. Cells were centrifuged and washed twice, the cell pellets resuspended in 100  $\mu$ l of anti-M13 antibody (5 Prime, 3 Prime Inc.) (diluted 1/400) and incubated for 45 min. Cells were washed as above, resuspended in 100  $\mu$ l of streptavidin-Phycoerythrin (Jackson Inc.) (diluted 1/400) and incubated for 20 min. After a final wash, the cells were analyzed by FACS.

### 6) Immunofluorescence

Cells were grown on coverslips to 50% confluency in 6 well-plates. Phage preparation (less than 10% of the culture medium) was added and the cells were incubated for 16 hours. The coverslips were washed 6 times with PBS, 3 times for 10 min with Glycine buffer (50 mM glycine, pH 2.8, NaCl 500 mM), neutralized with PBS and fixed with PBS-4% paraformaldehyde for 5 min at room temperature. Cells were permeabilized with cold acetone for 30 sec, saturated with PBS-1% BSA and incubated with anti-M13 antibody (d: 1/300 in the saturation solution) followed by streptavidin-Texas Red (Amersham) (d: 1/300 in the saturation solution). Coverslips were analyzed with an Axioskop fluorescent microscope (Zeiss).

### 7) Bacteriophage mediated cell infection

CsCl phage preparations were diluted at least 10 fold in cell culture medium, filtered through a 0.45  $\mu$ m filter and added to 30% to 80% confluent cells. After incubation, the cells were trypsinized, washed with FACS buffer and analyzed for GFP expression by FACS. In the experiments where MCF7 and SKBR3 were co-cultured, ErbB2 expression was quantitated by FACS using the anti-ErbB2 mouse mAb 4D5 which binds ErbB2 ECD (10  $\mu$ g/ml) (1 hr), biotinylated sheep anti-mouse immunoglobulins (Amersham) and streptavidin-Phycoerythrin.

### C) Results

#### 1) Internalization of ErbB2 binding monovalent and multivalent F5 phage particles by ErbB2 expressing cells

We isolated the anti-ErbB2 scFv-F5 from a library of scFv displayed on the surface of bacteriophage as fusions to pIII (Sheets *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95(11): 6157-6162) by selection on ErbB2 expressing SKBR3 breast tumor cells and recovery of infectious phage from within the cell (M. Poul *et al.*, manuscript in preparation). This selection strategy was employed to select scFv capable of undergoing endocytosis upon receptor binding. When the pHEN-F5 phagemid vector is rescued with VCS-M13 helper phage, the resulting virus particles (F5-phagemid) display an average of 1 copy of scFv-pIII fusion protein and 3 to 4 copies of the wild type pIII minor coat protein from the helper phage (Marks *et al.* (1992) *J. Biol. Chem.* 267(23): 16007-16010). As a result, the phagemid bind monovalently. To improve the binding of the virus particles to ErbB2 expressing cells, multivalent phage antibodies were created by subcloning the F5 scFv DNA into the phage vector fd-Sfi/Not for fusion with the pIII protein. Virus particles, referred to as fd-F5 phage, display 4 to 5 copies of scFv-pIII fusion protein (*Id.*).

To determine whether F5 phage antibodies could be internalized by mammalian cells, SKBR3 cells overexpressing ErbB2 were incubated for 16 hrs with fd-F5 phage ( $10^9$  colony forming unit/ml, cfu/ml), F5 phagemid ( $10^{11}$  cfu/ml), or with phagemids displaying an irrelevant anti-botulinum scFv-pIII fusion protein ( $10^{12}$  cfu/ml) (Amersdorfer *et al.*, 1997) as a negative control. The cell surface was stripped of phage antibodies using low pH glycine buffer, the cells permeabilized and fixed, and intracellular phage detected with anti-M13 antibody. Remarkably, all cells showed strong intracellular staining when incubated with fd-F5 phage or with F5 phagemid but not when incubated with the anti-botulinum phagemid. This demonstrates the dependence of phage entry on the specificity of the scFv fused to pIII.

#### 2) Preparation of ErbB2 binding phages and phagemids packaging a reporter gene for expression in eukaryotic cells

Two strategies were used to investigate whether F5 phage could deliver a reporter gene to mammalian cells leading to expression. To make monovalent phage

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containing a reporter gene, we cloned the gene for green fluorescent protein (GFP) driven by the CMV promoter into the phagemid vector pHEN-F5 generating the vector pHEN-F5-GFP (Figure 6, left panel). *Escherichia coli* TG1 containing pHEN-F5-GFP (ampicillin resistant) were infected with helper phage (kanamycin resistant) and high titers of monovalent F5-GFP phagemids were obtained ( $5.0 \times 10^{10}$  ampicillin resistant cfu/ml of culture supernatant). The ratio of packaged phagemid DNA versus helper phage DNA (ampicillin versus kanamycin resistant cfu) was determined to be 100:1. To make multivalent phage containing a reporter gene, fd-F5-GFP phage were generated by infecting *E. coli* TG1 carrying the pcDNA3-GFP phagemid (ampicillin resistant) with fd-F5 phage (tetracycline resistant), thus using fd-F5 phage as a helper phage. The fd-F5-GFP phage titer was approximately  $5.0 \times 10^8$  ampicillin resistant cfu/ml of culture supernatant. Lower phage titers result when fd is used as a helper phage because it lacks a plasmid origin of replication leading to interference from the phagemid fl origin (Cleary and Ray (1980) *Proc. Natl. Acad. Sci. USA* 77(8): 4638-4642). The ratio of packaged reporter gene DNA versus phage DNA (ampicillin versus tetracycline resistant cfu) was 1:1. The lower ratio of reporter gene/helper genome when using fd as a helper phage is due to the presence of a fully functional packaging signal on the fd genome compared to the mutated packaging signal in VCS-M13 (Vieira and Messing (1987) *Meth. Enzymol.* 153: 3-11). Both phage and phagemid preparations were assessed for SKBR3 cell binding (Figure 7). While both preparations bound SKBR3 cells, binding could be detected with as little as  $10^8$  cfu/ml of fd-F5-GFP phage cfu/ml (160 femtomolar) compared to  $10^{10}$  cfu/ml of F5-GFP phagemids (15 picomolar). Thus multivalent binding leads to an increase in the apparent binding constant of virus particles.

### 3) Targeted phagemid and phage mediated gene transfer into ErbB2 expressing breast cancer cells

To determine if ErbB2 binding phagemids were capable of targeted gene delivery,  $2.0 \times 10^5$  SKBR3 cells (a breast tumor cell line expressing high levels of ErbB2) or  $2.0 \times 10^5$  MCF7 cells (a low ErbB2 expressing breast tumor cell line) were incubated with  $5.0 \times 10^{11}$  cfu/ml F5-GFP phagemids at 37°C. Cells were analyzed for GFP expression by FACS after 48 hrs (Figure 8A). 1.37% of the SKBR3 cells expressed GFP after incubation with F5-GFP phagemids (Figure 8A6). GFP expression was identical regardless of the orientation of the fl packaging signal (data not shown), indicating that

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transcription/translation was proceeding *via* synthesis of the complementary DNA strand. . . . GFP expression was not detected in SKBR3 cells incubated with no phage or with helper phage packaging the reporter gene (Figure 8A4 and 8A5). Expression was also not seen in MCF7 cells incubated with no phage, helper phage or pHEN-F5-GFP, indicating the requirement of ErbB2 expression for targeted gene delivery (Figure 8A1, 8A2 and 4A3). Since gene transfer applications are likely to involve targeting of specific cells in an heterogeneous cell population, we performed the same experiment on a co-culture of SKBR3 and MCF7 cells (Figure 8B). Cells were stained for ErbB2 expression to discriminate MCF7 from SKBR3 cells and the GFP expression of each subpopulation was analyzed by FACS. Only SKBR3 cells (1.91%) expressed GFP. Similar results were found using F5-GFP phages instead of F5-GFP phagemids (data not shown). These data confirm that fd-F5-GFP phage and F5-GFP phagemid mediated gene delivery is restricted to ErbB2 overexpressing cells and can be targeted to such cells in the presence of non-expressing cells.

#### 4) Characterization of phage mediated gene transfer

To determine the dose-response characteristics of phage mediated gene transfer, SKBR3 cells were incubated for 60 hrs with increasing amounts of fd-F5-GFP phage or F5-GFP phagemids and the percent of GFP positive cells determined (Figure 9A and 9B). The minimal phage concentration required for detection of a significant number of GFP positive cells (Figure 9A) was approximately  $4.0 \times 10^7$  cfu/ml for fd-F5-GFP phage (0.13%) and  $1.0 \times 10^{10}$  cfu/ml for F5-GFP phagemid (0.12%). The values correlate closely with the binding curves (Figure 7) and indicate that multivalent phage are 100 to 1000 time more efficient than phagemids in terms of gene expression. No significant number of positive cells were observed with up to  $4.0 \times 10^{13}$  cfu/ml of helper phage packaging the reporter gene. For both phage and phagemid, the percent of GFP positive cells increased with phage concentration with no evidence of a plateau. The maximum values achieved were 2% of cells for fd-F5-GFP phage and 4% for F5-GFP phagemids and appear to be limited by the phage titer applied ( $1.5 \times 10^9$  cfu/ml and  $4.0 \times 10^{12}$  cfu/ml respectively). The amount of GFP expressed per cell (estimated by the mean fluorescent intensity (MFI), Figure 9B) also increased with phage concentration, with a small number of cells showing expression with phage titers as low as  $2.0 \times 10^7$  cfu/ml (fd-F5-GFP phage) to  $1.0 \times 10^{10}$  cfu/ml (F5-GFP phagemid).

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To compare the yield of gene expression obtained with phage to traditional transfection methods, single stranded (ssDNA) or double stranded (dsDNA) was transfected into SKBR3 using lipofectamine. Per  $\mu\text{g}$  of ss DNA, efficiency of phagemid mediated gene delivery (approximately 1%) was comparable to lipofectamine transfection of ssDNA (0.98%) and dsDNA (1.27%) (Table 10). Efficiency was approximately 500 fold higher for phage mediated transfection, with 2.25 ng of ss DNA resulting in transfection of 0.87% of cells.

Table 10. Transfection efficiencies in SKBR3 cells.

Transfection method	Reporter plasmid	Amount of reporter plasmid DNA	% of GFP positive cells*
F5-phagemid Mediated	pHEN-F5-GFP	15 $\mu\text{g}$	3.84
		3.1 $\mu\text{g}$	1.44
		0.78 $\mu\text{g}$	0.64
fd-F5-phage mediated	pcDNA3-GFP	5 ng	1.69
		2.25 ng	0.87
		1.25 ng	0.57
Helper phage mediated	pN2GFP	100 $\mu\text{g}$	0.12
		20 $\mu\text{g}$	0.07
		5 $\mu\text{g}$	0.06
Lipofectamine	pN2GFP	dsDNA 1 $\mu\text{g}$	1.27
		ssDNA 1 $\mu\text{g}$	0.98

\*Cells were analysed 48 hours after transfection for GFP expression using FACS. Results are expressed in % of GFP positive cells. \*\*For phage, the amount of reporter plasmid was calculated from the plasmid size and the number of ampicillin (pHEN-F5-GFP or pcDNA3-GFP) or kanamycin (pN2GFP) resistant colonies. Mock transfected cells contained an average of 0.05% GFP positive cells.

15

To determine the time course of gene expression,  $5.0 \times 10^{11}$  cfu/ml of F5-GFP phagemid were incubated with SKBR3 cells. After 48 hrs, the culture medium was replaced by fresh medium. GFP expressing cells can be detected within 24 hrs after phage are applied

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and the percentage of positive cells increases linearly with increasing time to a maximum of 4.5% by 120 hours (Figure 9C). The GFP content of the positive cells, as estimated by the MFI, increases up to 96 hrs (Figure 9D). After 96 hrs, the number of GFP positive cells continues to increase but the MFI decreases, probably due to the repartition of GFP molecules to daughter cells during cell division.

### C) Discussion

We demonstrate that filamentous phage displaying an anti-ErbB2 scFv antibody fragment as a genetic fusion with the minor coat protein pIII can be directly targeted to mammalian cells expressing the specificity of the scFv. Such phage undergo receptor mediated endocytosis and enter an intracellular trafficking pathway which ultimately leads to reporter gene expression. This is a remarkable finding demonstrating that prokaryotic viruses can be re-engineered to infect eukaryotic cells resulting in expression of a portion of the bacteriophage genome. Gene expression was detected with as few as  $2.0 \times 10^7$  cfu of phage and increased with increasing phage titer up to 4% of cells. Multivalent display decreased the threshold for detectable gene expression approximately 500 fold compared to monovalent display, most likely due to an increase in the functional affinity and an increased rate of receptor mediated endocytosis from receptor crosslinking. The maximum percent of cells transfected, however, was higher for monovalent display (phagemid) due to the significantly higher phage titer generated. The lower titer of multivalent phage is due to interference of the fl origin of replication on the reporter phagemid with the fd phage antibody origin of replication (Cleary and Ray (1980) *Proc. Natl. Acad. Sci. USA* 77(8): 4638-4642).

Targeted infection of mammalian cells using phage which bind endocytosable receptors is likely to be a general phenomenon. For example, fusing an anti-transferrin receptor scFv to gene III of pHEN-GFP results in GFP expression in 10% of MCF7 cells, 4% of SKBR3 cells, 1% of LNCaP cells and 1% of primary melanoma cells. Similarly, targeted GFP gene delivery to FGF receptor expressing cells using biotinylated phage and a streptavidin-FGF fusion molecule was recently reported (Larocca *et al.* (1998) *Hum. Gene Ther.* 9: 2393-2399). However, direct genetic fusion of the targeting molecule via gene III may be more efficient than using adapter molecules. Thus while the maximum percent of cells transfected using the FGF-adapter molecule was not reported, we estimate it to be only 0.03% of FGF expressing L6 rat myoblasts based on the number of cells infected, the time

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after infection to the measurement of gene expression and the number of cells expressing GFP. While a greater frequency of expression (0.5%) was seen in COS-1 cells, this results from the presence of large T antigen and SV40 mediated DNA replication and thus is not generalizable to most cells.

5           The approach we describe represents a novel method to discover ligands for targeted intracellular drug or gene delivery. Phage antibody or peptide libraries are first selected for endocytosis by mammalian cells (Barry *et al.* (1996) *Nat. Med.* 2: 299-305) or for binding to purified antigen, cells, tissues or organs. After subcloning the selected scFv genes into the pHEN-GFP vector, phage produced from individual colonies can be directly  
10   screened for gene expression. This is possible since expression can be detected with as little as  $1.0 \times 10^{10}$  cfu of phagemids. This permits not only direct identification of endocytosed scFv but also the subset of receptor antibodies which undergo proper trafficking for gene expression. If multivalent display is necessary for efficient endocytosis, the scFv genes can be subcloned into fd-Sfi-Not which is then used to rescue the reporter phagemid. Use of  
15   scFv-fd phage also allows the targeting of a large number of different reporter genes in various expression vectors since many commercially available mammalian vectors contain fl origins of replication. As such, antibody targeted phage might prove useful transfection reagents, especially for cells difficult to transfect by standard techniques.

          It may also prove possible to use this approach to directly select, rather than  
20   screen, antibodies for targeted gene delivery. For example, mammalian cells are incubated with a phage antibody library containing the GFP gene, and then sorted based on GFP expression using FACS. Phage antibody DNA would be recovered from the mammalian cytoplasm by cell lysis and used to transfect *E. coli* and prepare more phage for another round of selection. If the quantities of recoverable phage DNA are inadequate, inclusion of  
25   the neomycin gene in the pHEN-GFP vector would permit selection of GFP expressing mammalian cells using G418 (Larocca *et al. supra*).

          Finally, this system has promise as a targetable *in vitro* or *in vivo* gene therapy vehicle. The main limitations are infection efficiency, pharmacokinetics and immunogenicity. With respect to infection efficiency, values achieved by targeted phage in  
30   this report ( $8.0 \times 10^4$ /ml of phage preparation) are not dissimilar to values reported for targeted retrovirus ( $10^3$ - $10^5$ /ml of virus) (Kasahara *et al.* (1994) *Science* 266: 1373-1376; Somia *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92(16): 7570-7574) but less than reported



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for adenovirus targeting strategies (Douglas *et al.* (1996) *Nat. Biotechnol.* 14: 1574-1578; Watkins *et al.* 1997) *Gene Ther.* 4(10): 1004-1012). The factors limiting higher infection efficiencies, however, are likely to differ between the systems. Thus while the percentage of cells infected by retrovirus is significantly higher than observed for bacteriophage, infection is limited by the problems encountered producing large numbers of virus which can enter the cell. Since all cells take up the targeted phage, gene expression is limited by one or several post-uptake events (*e.g.* degradation of phage to release DNA, endosomal escape, nuclear targeting or transcription). More detailed study of the fate of the phage and its DNA is likely to suggest where the block lies permitting engineering of phage to increase infection efficiency. For example, endosomal escape could be enhanced by co-administering replication defective adenovirus (Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88(19): 8850-8854) or incorporating endosomal escape peptides (Wagner *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89(17): 7934-7938) or proteins (Fominaya and Wels (1996) *J. Biol. Chem.* 271(18): 10560-10568) into the phage major coat protein pVIII. Alternatively, infection efficiency could be increased combinatorially by creating scFv targeted libraries of pVIII mutants and selecting for increased gene expression. With respect to pharmacokinetics, though not extensively studied, it is likely that the biodistribution of phage is limited to the intravascular space. This would not affect *in vitro* phage gene therapy, but might limit *in vivo* uses to those targeting the vasculature. This still leaves numerous applications including those where neovascularization plays a role, such as cancer. With respect to immunogenicity, it is likely that phage will be immunogenic, thus limiting the number of times that phage could be administered *in vivo*. Alternatively, it might prove possible to evolve the major coat protein pVIII to reduce or eliminate immunogenicity for example by negatively selecting a pVIII library on immune serum (Jenne *et al.* (1998) *J. Immunol.* 161(6): 3161-3168).

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

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## CLAIMS

What is claimed is:

- 1                   1.     A method of transfecting a target cell with a heterologous nucleic  
2     acid, said method comprising:  
3                    i) providing a phage externally displaying a heterologous targeting  
4     protein and containing said heterologous nucleic acid; and  
5                    ii) contacting said target cell with said phage whereby said phage is  
6     internalized into said cell and wherein said heterologous nucleic acid is transcribed within  
7     said cell.
- 1                   2.     The method of claim 1, wherein said phage displays, on average, at  
2     least two copies of said heterologous targeting protein.
- 1                   3.     The method of claim 2, wherein said phage displays, on average, at  
2     least four copies of said heterologous targeting protein.
- 1                   4.     The method of claim 1, wherein said phage is a member of a library of  
2     phage wherein said library comprises a number of different heterologous targeting proteins.
- 1                   5.     The method of claim 4, wherein said library comprises at least about  
2     10<sup>5</sup> different heterologous targeting proteins.
- 1                   6.     The method of claim 4, further comprising selecting phage that are  
2     internalized by said cell.
- 1                   7.     The method of claim 6, wherein said selecting is by expression of a  
2     selectable marker or a reporter gene.
- 1                   8.     The method of claim 7, wherein said selectable marker is an antibiotic  
2     resistance gene.
- 1                   9.     The method of claim 7, wherein said reporter gene is selected from the  
2     group consisting of Fflux,  $\beta$ -galactosidase, horse radish peroxidase, and green fluorescent  
3     protein.

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- 1                   10.    The method of claim 6, further comprising amplifying phage  
2 internalized by said cell.
- 1                   11.    The method of claim 1, wherein said target cell is a mammalian cell.
- 1                   12.    The method of claim 1, wherein said providing comprises:  
2                    i) providing an assembly cell containing said heterologous nucleic  
3 acid and a packaging signal; and  
4                    ii) infecting said assembly cell with a phage expressing on its surface  
5 said heterologous targeting protein and containing the gene for said targeting protein  
6 whereby said phage acts as a helper phage and packages said heterologous nucleic acid.
- 1                   13.    The method of claim 12, wherein said assembly cell is a prokaryote.
- 1                   14.    The method of claim 1, wherein said heterologous targeting protein, a  
2 DNA encoding said heterologous targeting protein, and said heterologous nucleic acid are  
3 encoded by a DNA that is a phagemid.
- 1                   15.    The method of claim 1, wherein said heterologous targeting protein is  
2 encoded by a nucleic acid that is a phagemid.
- 1                   16.    The method of claim 1, wherein said phage is a filamentous phage.
- 1                   17.    The method of claim 1, wherein said targeting protein is an antibody.
- 1                   18.    The method of claim 17, wherein said antibody is a single chain Fv  
2 (scFv) or a Fab.
- 1                   19.    The method of claim 18, wherein said antibody is a single-chain Fv  
2 (scFv).
- 1                   20.    The method of claim 1, wherein said targeting protein is an anti-erbB2  
2 antibody and said phage is a filamentous phage.
- 1                   21.    The method of claim 1, wherein said phage is preselected for binding  
2 to an internalizing cell surface receptor.

- 1                   22.    The method of claim 21, wherein said receptor is selected from the  
2   group consisting of a transferrin receptor, erbB2, EGF receptor, and Vegf receptor.
- 1                   23.    The method of claim 1, wherein said phage further expresses an  
2   endosomal escape polypeptide.
- 1                   24.    The method of claim 1, wherein said phage further comprises anuclear  
2   localization signal.
- 1                   25.    The method of claim 23, wherein said endosomal escape polypeptide  
2   is a bacterial translocation domain or a viral endosomal escape peptide.
- 1                   26.    A vector for transfection of a target cell, said vector comprising a  
2   phage displaying a heterologous targeting protein that specifically binds to an internalizing  
3   receptor whereby said phage binds to and is internalized into said target cell, and wherein  
4   said phage contains a heterologous nucleic acid that is transcribed inside said target cell.
- 1                   27.    The vector of claim 26, wherein said heterologous nucleic acid is not  
2   drug resistance gene.
- 1                   28.    The vector of claim 26, wherein said heterologous nucleic acid is not a  
2   selectable marker.
- 1                   29.    The vector of claim 26, wherein said heterologous targeting protein is  
2   an antibody.
- 1                   30.    The vector of claim 29, wherein said antibody is a single chain Fv  
2   (scFv), or a Fab.
- 1                   31.    The vector of claim 30, wherein said antibody is a single-chain Fv.
- 1                   32.    The vector of claim 26, wherein said phage is a filamentous phage.
- 1                   33.    The vector of claim 26, wherein said heterologous targeting protein is  
2   present on average in at least two copies per phage.

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1                   34.    The vector of claim 33, wherein said heterologous targeting protein is  
2 present on average in at least four copies per phage.

1                   35.    A vector for transfection of a target cell, said vector comprising a  
2 phage vector or phagemid vector encoding:  
3                   a phage coat protein in fusion with a heterologous targeting protein  
4 that specifically binds to an internalizing cell surface receptor and is internalized into a cell  
5 bearing said receptor, and  
6                   a heterologous nucleic acid in an expression cassette allowing  
7 transcription of said heterologous nucleic acid inside said cell..

1                   36.    The vector of claim 35, wherein said heterologous nucleic acid is not  
2 drug resistance gene.

1                   37.    The vector of claim 35, wherein said heterologous nucleic acid is not a  
2 selectable marker.

1                   38.    The vector of claim 35, wherein said heterologous targeting protein is  
2 an antibody.

1                   39.    The vector of claim 38, wherein said antibody is a single chain Fv  
2 (scFv), or a Fab.

1                   40.    The vector of claim 39, wherein said antibody is a single-chain Fv.

1                   41.    The vector of claim 35, wherein said vector, when packaged into a  
2 filamentous phage, displays on average at least two copies of said heterologous targeting  
3 protein per phage particle.

1                   42.    A kit for transducing a target cell, said kit comprising a container  
2 containing a phage or phagemid vector encoding:  
3                   a phage coat protein in fusion with a heterologous targeting protein  
4 that specifically binds to an internalizing cell surface receptor and is internalized into a cell  
5 bearing said receptor, and

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6 a pair of restriction sites that allow insertion of a heterologous nucleic  
7 acid into said phage or phagemid vector.

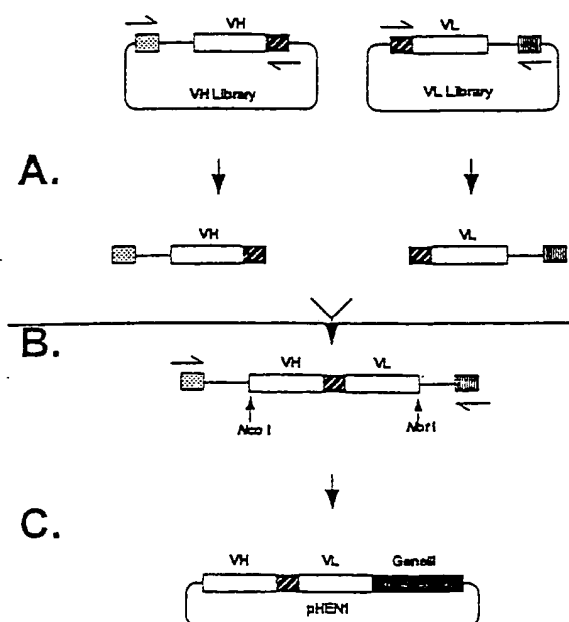
1 43. The vector of claim 42, wherein said heterologous targeting protein is  
2 an antibody.

1 44. The vector of claim 43, wherein said antibody is a single chain Fv  
2 (scFv), or a Fab.

1 45. The vector of claim 44, wherein said antibody is a single-chain Fv.

1 46. The vector of claim 42, wherein said vector, when packaged into a  
2 filamentous phage, displays on average at least two copies of said heterologous targeting  
3 protein per phage particle.

1 / 9

*Fig. 1*

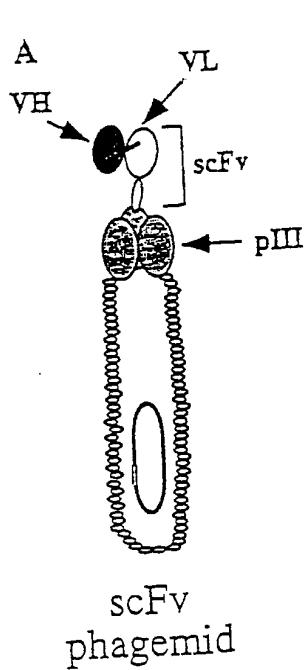


Fig. 2A

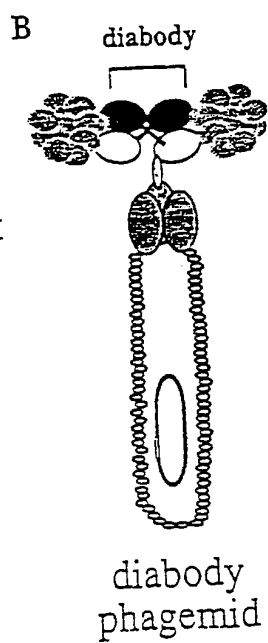


Fig. 2B

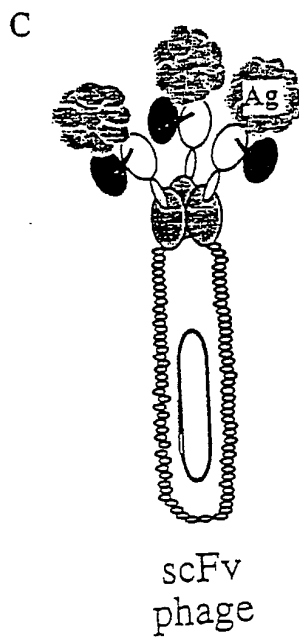


Fig. 2C



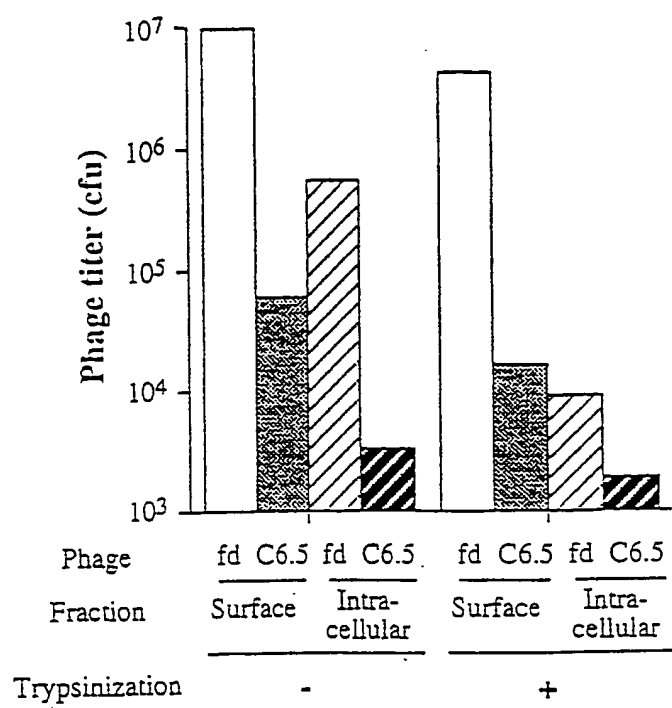


Fig. 3

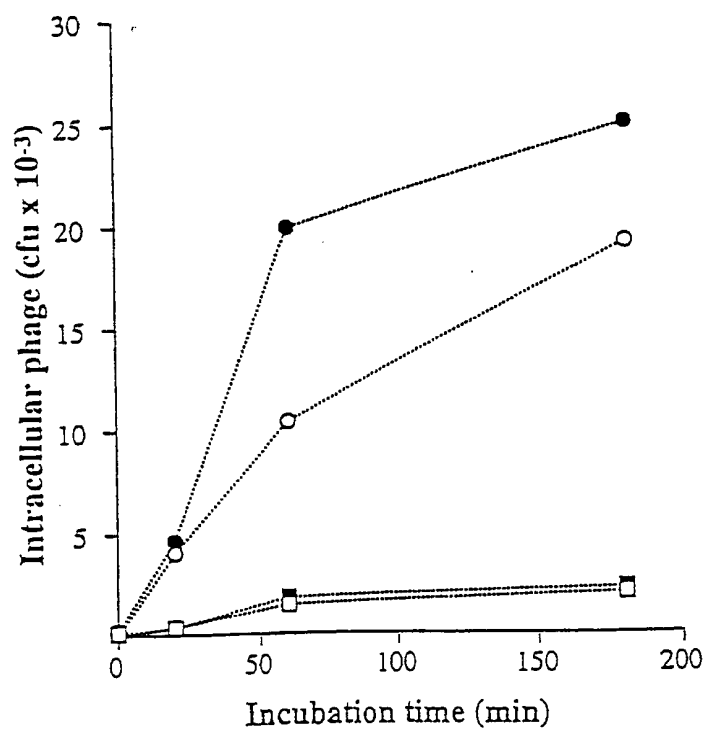


Fig 4

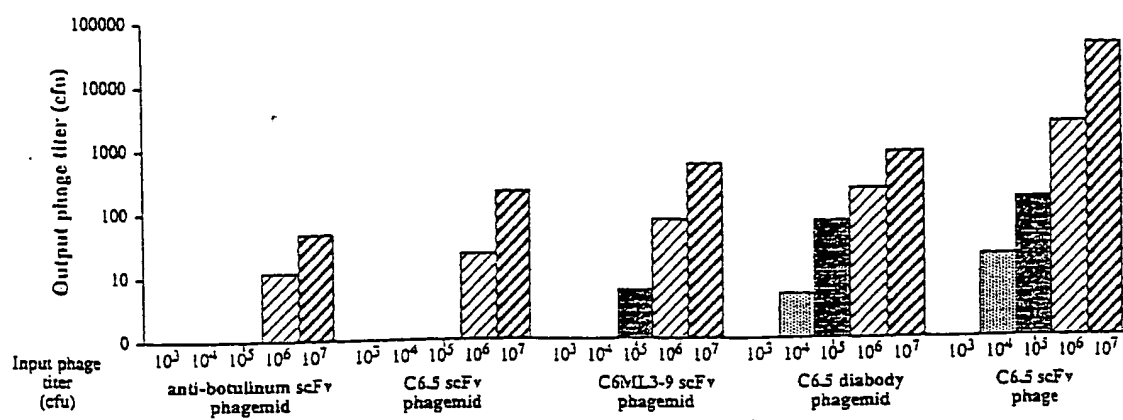


Fig. 5

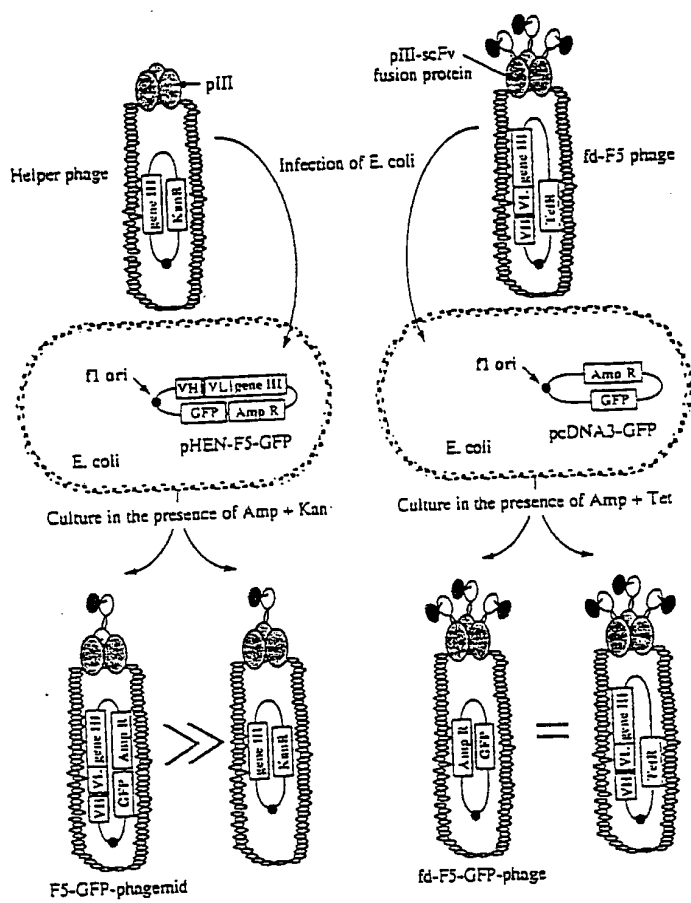


Fig. 6

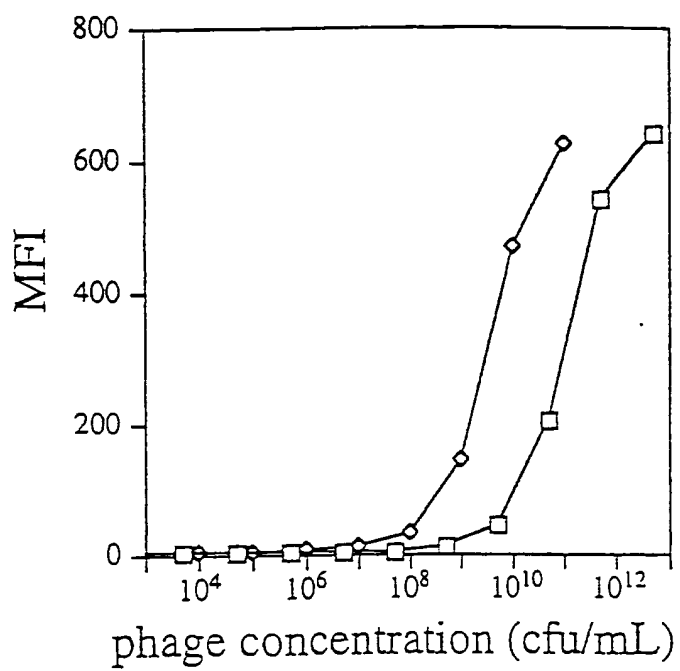


Fig. 7

Fig. 8A

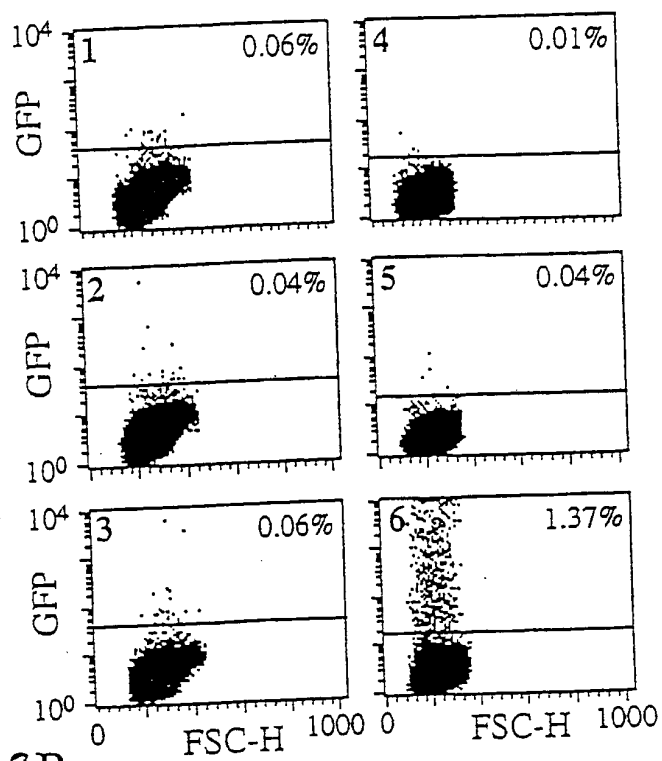


Fig. 8B

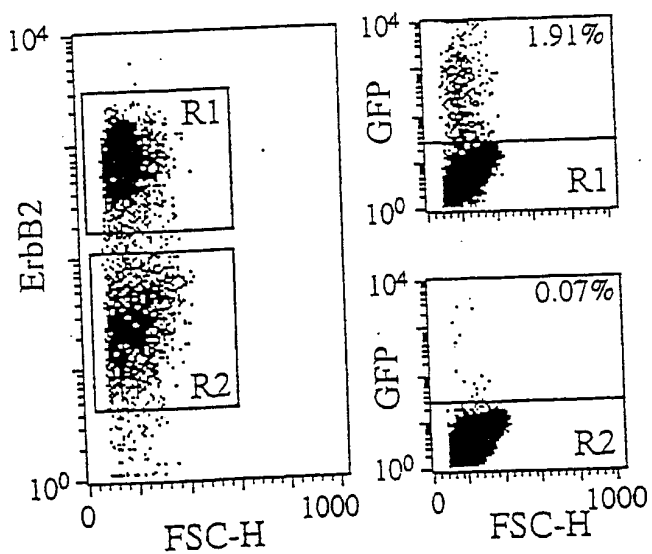


Fig 9A

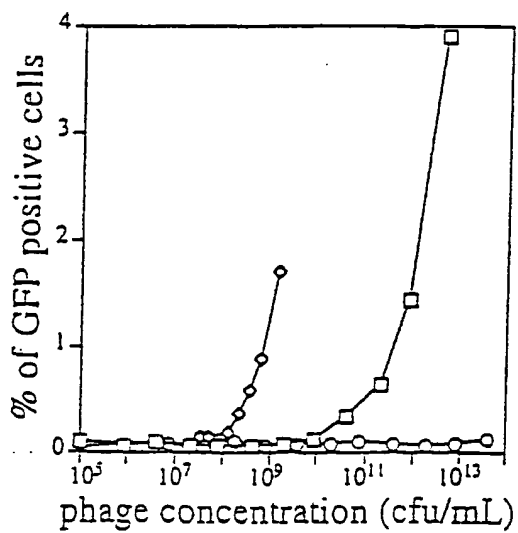


Fig. 9B

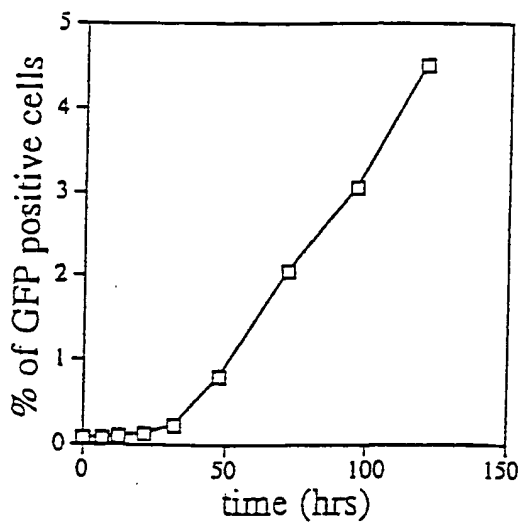
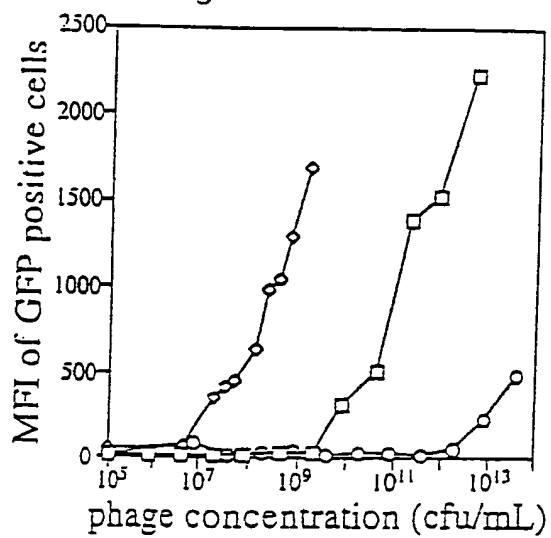


Fig 9C

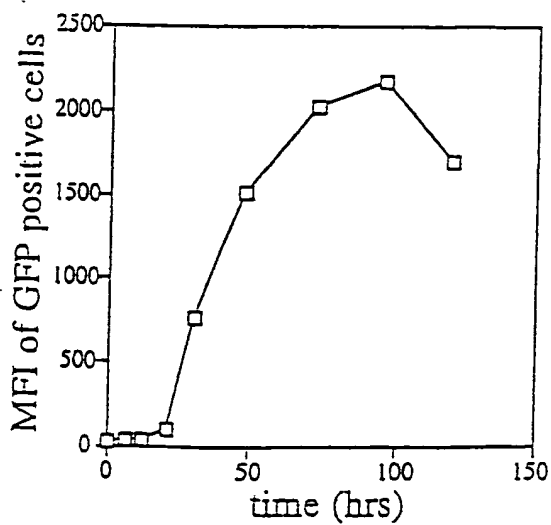


Fig 9D

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/07398

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12N 7/00, 7/04, 15/63

US CL : 435/69.1, 235.1, 236, 320.1, 440; 536/23.1, 23.4, 23.5, 23.72

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 235.1, 236, 320.1, 440; 536/23.1, 23.4, 23.5, 23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, INPADOC

search terms: phage? viral? virus? displa? transfect? transform? phagemid? target? internal? librar?

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	US 5,885,793 A (GRIFFITHS et al.) 23 March 1999, abstract, col. 5, lines 34-53, col. 8, lines 5-21	1-46
P, X	LAROCCA et al. Gene Transfer to Mammalian Cells Using Genetically Targeted Filamentous Bacteriophage. The Faseb Journal. April 1999, Vol. 13, No. 6, pages 727-734, especially the abstract, page 729, Figure 1, page 732, Figure 4.	1-46

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 JUNE 1999

Date of mailing of the international search report

17 AUG 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer:

NANCY J. DEGEN

Telephone No. (703) 308-0196